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Two internal pools of soluble polyphosphate in the cyanobacterium *Synechocystis* sp. strain PCC 6308: an in vivo 31P NMR spectroscopic study

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Abstract Two intracellular pools of soluble polyphosphate were identified by in vivo ^{31}P NMR spectroscopy in the cyanobacterium *Synechocystis* sp. strain PCC 6308. Polyphosphate was present in the cells after growth in sulfur-limited media containing excess phosphate. The presence of polyphosphate was confirmed by transmission electron microscopy and chemical analysis. ^{31}P NMR spectroscopy of whole cells treated with EDTA revealed two pools of mobile polyphosphate. A downfield shift and narrowing of part of the broad polyphosphate resonance was observed after EDTA treatment, suggesting that EDTA binds metal ions normally associated with some of the polyphosphate. Phosphate, but not polyphosphate, leaked out of the cells after this treatment. Addition of magnesium ions caused the downfield shift in the polyphosphate resonance to move back toward its original value. These data show that only part of the cation-complexed polyphosphate is accessible to the added EDTA and suggest that there are two internal fractions of NMR-visible polyphosphate in the cells, only one of which loses its associated cations to EDTA. Spheroplast formation showed that polyphosphate was not present in the periplasm of the cells.

Key words Cyanobacteria · Polyphosphate · In vivo ^{31}P NMR spectroscopy · *Synechocystis* sp. strain PCC 6308 · EDTA permeabilization; Spheroplasts

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Abbreviations P_i Inorganic phosphate · *PolyP* Polyphosphate · MDPA Methylene diphosphonic acid

Introduction

Cyanobacterial growth is dependent upon a number of essential chemical elements obtained from the environment surrounding the cell. One of these is phosphorus, which is present in nucleic acids, phospholipids, lipopolysaccharides, and cytoplasmic solutes. When excess environmental phosphorus is available in the form of inorganic phosphate (P_i), cells often sequester P_i in the form of inorganic polyphosphate (polyP), a linear polymer of three to more than a thousand P_i residues linked by high-energy phosphoanhydride bonds (Wood and Clark 1988). Non-membrane-bound polyP bodies, or volutin, have been observed in cyanobacteria when cells are starved for an essential nutrient in the presence of large amounts of P_i. For example, Lawry and Jensen (1979) have shown that sulfur deficiency causes increases in the amount of cellular phosphorus and of polyP bodies. Through the use of in situ X-ray energy dispersive analysis, the polyP granules in *Plectonema boryanum* have been found to contain large amounts of P and K and small amounts of Ca and Mg (Sicko-Goad et al. 1975; Baxter and Jensen 1980). P_i sequestered as polyP can be used when P_i is limiting in the environment (Stewart et al. 1978) and may act as an energy reserve (Harold 1966; Kulaev and Vagabov 1983; Wood and Clark 1988). Kornberg (1995) has recently discussed numerous biological functions performed by polyP and its importance in nature and for practical and theoretical applications.

Little information is available on phosphorus regulation in cyanobacteria, although phosphate uptake and the activities of the enzymes polyP synthetase and polyphosphatase have been determined under various growth conditions in the cyanobacterium *Synechococcus* (Grillo and Gibson 1979). The activity of polyP synthetase in cell extracts increased 20-fold during phosphate starvation, but polyphosphatase activity changed little during growth un-

der the different conditions tested. Interestingly, polyP did not turn over during unrestricted growth and was not selectively mobilized during phosphate starvation.

In vivo NMR spectroscopy has become a useful tool for studying cellular metabolism since it is nondestructive and enables the performance of time course experiments and investigations of intact cells (Shulman et al. 1979; Roberts and Jardetzky 1981). In cyanobacteria, in vivo ^{31}P NMR spectroscopy has given information about a number of intracellular phosphate metabolites (Lawrence et al. 1997), about the bioenergetics of the cell, and about the pH within the cell (Roberts 1984; Kallas and Dahlquist 1981). ^{31}P NMR spectroscopy is used as a metabolic monitor in cyanobacterial studies to accompany other forms of NMR spectroscopy such as ^{15}N observations (Callies et al. 1992; Walter et al. 1992). The work reported here began when a large amount of polyP was observed during NMR spectroscopy studies of nutrient-stressed *Synechocystis* sp. strain PCC 6308. Although some polyP is observed in many cyanobacteria even during exponential growth, it had not been previously observed in this strain. The objective of this investigation, therefore, was to study the behavior of polyP within sulfur-starved, phosphate-enriched cells of *Synechocystis* sp. strain PCC 6308 using ^{31}P NMR spectroscopy of EDTA-treated cells and of spheroplasts, transmission electron microscopy, and chemical analysis. NMR spectroscopy detects soluble, mobile polyP; electron microscopy allows visualization of insoluble polyP in polyP bodies; and chemical analysis measures both soluble and insoluble polyP.

Materials and methods

Organism and growth conditions

The unicellular, non-nitrogen-fixing cyanobacterium *Synechocystis* sp. strain PCC 6308 (ATCC 27150) was grown routinely at 35° C in liquid medium BG-11 (Allen 1968a) supplemented with Na_2CO_3 (2.4 g/l). Roux flasks containing cyanobacteria were bubbled with 5% CO_2 in air under cool white fluorescent light intensity of 100 $\mu E\ m^{-2}\ s^{-1}$. These were considered standard conditions. Phosphorus-free medium was prepared using liquid medium BG-11 containing Hepes (40 mg/l) in place of K_2HPO_4. Sulfur-free, tenfold-phosphorus-enriched medium was prepared using BG-11 containing $MgCl_2 \cdot 6\ H_2O$ (61.8 mg/l) in place of $MgSO_4 \cdot 7H_2O$ and additional K_2HPO_4 (351 mg/l). Growth was routinely assayed by measuring optical density at 750 nm with a Hitachi U-200 spectrophotometer.

For NMR analysis, cells were inoculated from exponentially growing cells into new media and were allowed to grow for 48 h. The cells were harvested by centrifugation (Dupont Sorvall RC-5B centrifuge, GSA rotor; 10,400 × *g*, 5 min) and washed twice with phosphorus-free BG-11 medium. The resultant pellet was kept on ice for 1–2 h before resuspension for NMR measurements. In all cases, the optical density and wet or dry weight were determined. Pellet dry weights ranged from 98 to 420 mg for exponentially grown cells and 70 to 105 mg for sulfur-depleted, phosphorus-enriched cells.

Spheroplast formation

Cells were treated for 2 h at room temperature with a solution containing 330 mM sorbitol, 50 mM Hepes, 2.5 mg lysozyme/ml, and 10 mM EDTA according to the protocol of Yang et al. (1994). Cell suspensions and supernatants produced after centrifugation for 10 min in a table centrifuge were analyzed by light microscopy, absorption spectrophotometry, and ^{31}P NMR spectroscopy. Pelleted spheroplasts were resuspended in either distilled water or Hepes and were allowed to lyse. The supernatant and resuspended pellet were analyzed by the same methods. Spheroplast formation conditions were optimized with respect to concentrations of lysozyme, EDTA, and sorbitol as well as with respect to incubation times and temperatures before the experiments described below were carried out.

^{31}P nuclear magnetic resonance spectroscopy

^{31}P NMR experiments were performed using a GE Omega 400 wide-bore NMR spectrometer at Brigham and Women's Hospital in Boston, Mass., USA. The 89-mm bore superconducting magnet (Oxford Spectrospin, UK) was equipped with a high-resolution 20-mm probe resonating at 161.94 MHz for ^{31}P, and a variable temperature accessory. Spectra were processed on a SUN 3 workstation running NMR1 software from New Methods Research (East Syracuse, N.Y., USA).

For low-temperature studies, the probe was cooled to 4° C before introduction of the sample tube. Otherwise, spectroscopy was performed at ambient magnet temperature, approximately 21° C. Because no time-dependent differences were observed in the spectra at the higher, ambient temperature, most of the spectra were taken at ambient temperature for convenience. The magnetic field was shimmed on the free induction decay of the water protons. Typical proton line widths were 60 Hz. ^{31}P NMR spectra were acquired using a 45° pulse width (16 μs), a 0.4-s pulse delay, 10,000–15,000 Hz spectral width, and 1,024 data points. Proton decoupling was not used. Because these are in vivo spectra with relatively broad ^{31}P lines (40–100 Hz), spectra were run unlocked. Generally, 2,000 acquisitions were carried out for each spectrum. Spectra were processed using an exponential multiplication factor of 20 Hz.

For NMR measurements, cells were resuspended to 10-13 ml total volume in phosphorus-free BG-11 medium or 0.05M Hepes (pH 8.0) and were placed in a clean 20-mm (outer diameter) NMR tube (Wilmad Glass, Buena, N.J., USA) along with a capillary containing an aqueous solution of methylene diphosphonic acid (MDPA, Sigma) to which chemical shifts were referred. The ^{31}P chemical shift of the methylene diphosphonic acid solution was independently determined to be 16.73 ppm downfield from 85% H_3PO_4. No attempt was made to maintain growth conditions using light or bubbling of cells in these experiments.

In experiments that included the addition of EDTA, 1 M Na_4EDTA was added in microliter amounts to a final concentration of up to 20 mM. Medium pH was essentially constant and greater than pH 8.0 after the first addition of EDTA.

Electron microscopy methods

Cells were prefixed in 1% osmium tetroxide (OsO_4) in veronal-acetate buffer after resuspension in tryptone media, as previously described (Kellenberger et al. 1958; Allen 1968b). They were then fixed for 4 h in 1% OsO_4 and stained in uranylacetate. After fixation and dehydration, cells were embedded in Spurr low-viscosity embedding resin (Polysciences), and sections were post-stained in uranylacetate and lead citrate. Sections were viewed with a Zeiss EM9S-2 electron microscope.

Chemical assay of polyP

PolyP was extracted from cells and hydrolyzed using an alkaline hypochlorite reagent as described by Poindexter and Eley (1983). Phosphate assays were performed according to Onishi (1975).

Results

A ^{31}P NMR spectrum, taken at 4° C, of a suspension of cells grown in BG-11 and then concentrated and resuspended in phosphorus-free media is presented in Fig. 1. The assignment of peaks was made according to published results (Kallas and Dahlquist 1981; Rao et al. 1985; Altenburger et al. 1991) and our own results (Lawrence et al. 1997). Peaks due to monophosphates, inorganic phosphate (P_i), phosphoenolpyruvate (PEP), uridine diphosphoglucose (UDPG), and the adenosine di- and tri-phosphates are noted in Fig. 1. The presence of a β-ATP peak in the spectrum is a strong indication that the cells are metabolically viable, although the relatively large P_i peak suggests less than optimal conditions. The monophosphate peak is a result of signals from many sugar phosphates, several of which have been identified using phosphorus (and verified by proton) NMR spectroscopy (Lawrence et al. 1997). Among them are ribulose-1,5-bis-phosphate, glyceraldehyde-3-phosphate, and 3-phosphoglyceric acid.

An ambient-temperature ^{31}P NMR spectrum of cells grown in sulfur-deficient, phosphorus-enriched medium is shown in Fig. 2a (the MDPA reference peak is not shown). The broad peak observed at –23 ppm is due to polyP (Rao et al. 1985). The polyP peak in cells grown under these conditions ranged in width from 274 to 376 Hz. Cells were observed by NMR spectroscopy before (Fig. 2a) and following (Fig. 2b) the addition of 10 mmol EDTA. In the presence of this EDTA concentration, part of the polyP peak sharpened and shifted downfield (peak width, 258 Hz; downfield shift, 1.6 ppm) The P_i peak also shifted downfield (48 Hz; 0.3 ppm) and increased in intensity relative to the external standard MDPA. Both the polyP observed as the downfield peak and the P_i must therefore be accessible to EDTA. The supernatant contained P_i, but no detectable polyP (spectrum not shown).

The narrowing and downfield shift of part of the polyP signal after the addition of EDTA can be explained by the loss of complexed divalent cations to EDTA by a fraction of the polyP. The increase in P_i signal is caused by the breakdown of some cytoplasmic polyP to P_i. The appearance of P_i in the NMR spectrum of the supernatant is caused by P_i leaking out of the cell as the permeability of the outer membrane increased due to EDTA treatment. Repetitions of this experiment with higher concentrations of EDTA always produced a large P_i peak in the supernatant, but only in the cases of very high concentrations of cellular polyP was a relatively small polyP signal observed in the supernatant. This suggests that the majority of the polyP is of a size too large to pass out of the plasma membrane and the permeabilized outer membrane. Absorption spectra of the supernatant showed little or no phycocyanin to be present (data not shown), evidence that the cytoplasmic membrane remained intact.

EDTA titration of cells grown in sulfur-limiting, excess phosphorus medium was performed to an EDTA concentration of up to 20 mM, followed by centrifugation and resuspension in EDTA-free medium. The effects on the ^{31}P NMR spectra of the resuspended cells caused by the addition of several concentrations of EDTA and resuspen-

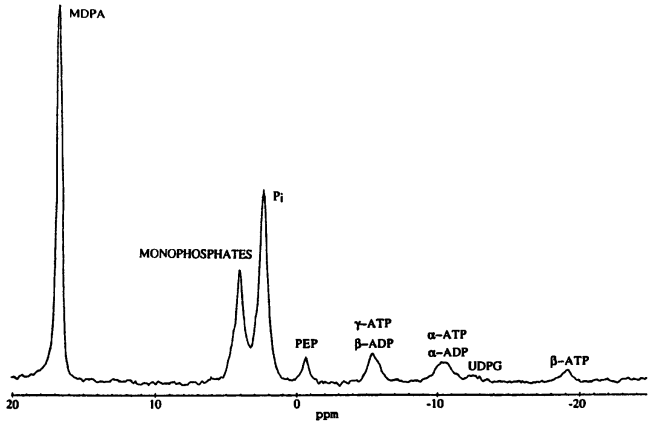

Fig. 1 In vivo ^{31}P NMR spectrum of logarithmically grown cells, with MDPA standard (16.73 ppm) shown. Data acquired at 4° C as described in Materials and methods

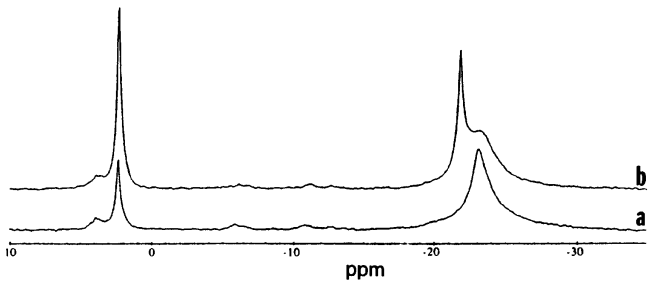

Fig. 2 Upfield regions of in vivo ^{31}P NMR spectra (MDPA reference peak at 16.73 ppm not shown) acquired at room temperature of **a** cells grown in sulfur-limited, excess phosphorus medium and **b** the same cells in 10 mM EDTA. Spectral parameters are described in Materials and methods

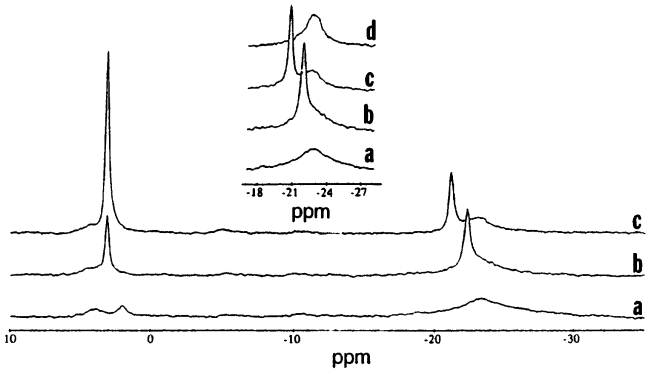

Fig. 3a–d Upfield regions of in vivo ^{31}P NMR spectra (MDPA reference peak at 16.73 ppm not shown) of cells grown in sulfur-limited, excess phosphorus medium, titrated with EDTA. Spectra were standardized to a constant MDPA peak area. **a** 0 mM EDTA, **b** 4 mM EDTA, and **c** 12 mM EDTA. The polyP region of the spectrum, shown in the *insert*, also includes **d** a spectrum of cells from **c** after centrifugation and resuspension in 7 mM $MgCl_2$. Spectral parameters are described in Materials and methods

sion of cells in $MgCl_2$ to a final concentration of 7 mM following centrifugation are shown in the spectra in Fig. 3. As the concentration of EDTA increased, a significant downfield shift and narrowing of the polyP peak, with the eventual resolution of a narrow downfield peak and a broad upfield peak, were observed. The effect of the EDTA on the chemical shift of the sharpened downfield polyP peak is plotted in Fig. 4. The chemical shift of this polyP peak moved downfield as EDTA concentration increased to 10 mM, and became constant at higher EDTA concentrations. All the cations that could be released from polyP were complexed by 10 mM EDTA. Addition of $MgCl_2$ caused

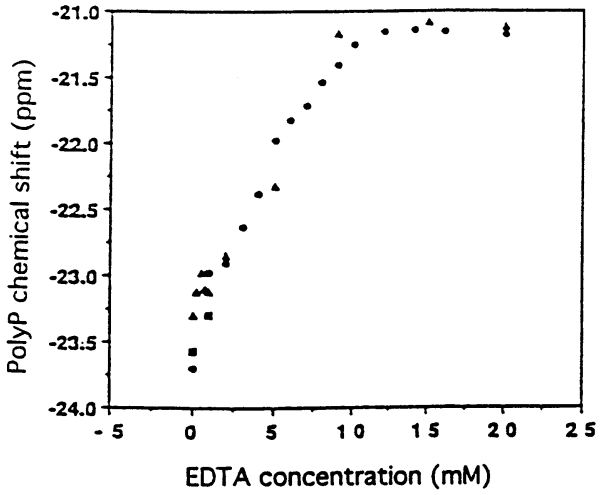

Fig. 4 The chemical shift of the downfield polyP peak as a function of EDTA concentration. *Different symbols* represent three separate experiments

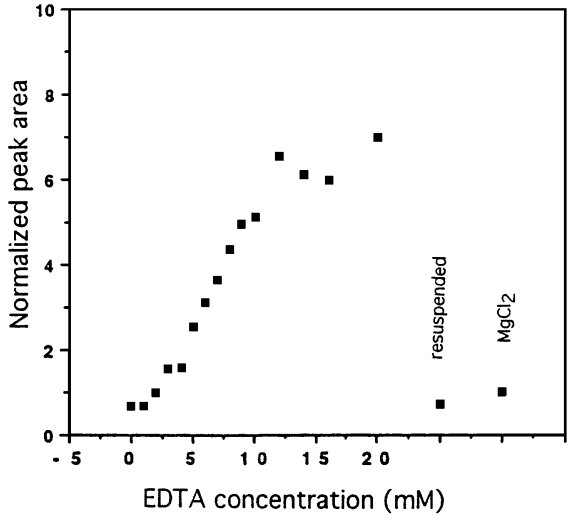

Fig. 5 The integrated area under the inorganic phosphate peak as a function of EDTA concentration. Cell suspensions were titrated with EDTA to a concentration of 20 mM, followed by centrifugation and resuspension in a medium 7 mM in $MgCl_2$. The area under the peaks was estimated using a curve-fitting program which fit the peak to a Lorentzian lineshape and normalized to the area of the MDPA reference peak. Spectral parameters are described in Materials and methods

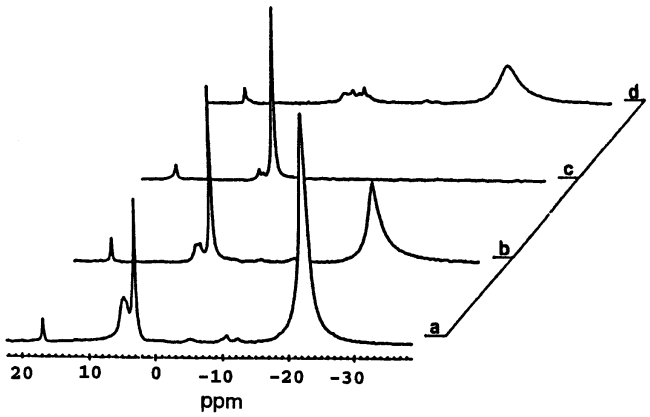

Fig. 6a–d ^{31}P NMR spectra of *Synechocystis* sp. strain PCC 6308 showing the effect of lysozyme-EDTA treatment forming spheroplasts and their subsequent lysis. Spectral intensities were standardized to a constant MDPA peak area. **a** Whole cells grown in sulfur-limited, excess phosphorus medium and suspended in BG-11 medium, **b** whole cells plus supernatant during lysozyme treatment, **c** periplasmic supernatant after spheroplasts were centrifuged, and **d** cytoplasmic supernatant after lysis of spheroplasts in double-distilled water. Spectral parameters are described in Materials and methods

the downfield-shifted, sharpened part of the polyP peak to shift back upfield toward its original position and to rebroaden. That fraction of the polyP that had lost its cations to EDTA now complexed with the added Mg^{2+}, and the single, broader signal was again observed.

In order to estimate changes in the amounts of the various phosphorus-containing compounds throughout the EDTA titration, the area under each of the peaks was estimated using a curve-fitting program after normalizing to the MDPA reference peak area. The total polyP peak area remained essentially constant throughout the titration, as did that of the sugar phosphates (data not shown). Only the P_i peak area, plotted in Fig. 5, changed significantly: it increased approximately sixfold as a function of EDTA concentration and dropped toward its original level after centrifugation and resuspension of the cells in 7 mM $MgCl_2$. This suggests that the P_i leaked out of the cytoplasm. A small breakdown in polyP would lead to the observed increase in the P_i peak area. Since much of the polyP is in granular form and is therefore not detectable by solution NMR methods, a corresponding decrease in the soluble polyP signal would not necessarily be observed.

Confirmation of the presence of polyP in the cells grown in sulfur-limited, phosphorus-excess medium was made by both transmission electron microscopy (not shown) and chemical analysis. Electron micrographs taken after fixation with 1% osmium tetroxide showed that nutritionally stressed cells exhibited large regions of low electron density that did not appear in the micrographs of exponentially grown cells. Since polyP bodies tend to be lost from thin-sections when the inclusions are large (Lawry and Jensen 1979), these regions, found only in the cytoplasm, were identified as evidence of granular, non-NMR-

detectable polyP. P_i analysis of alkaline hypochlorite-hydrolyzed cells showed that cells grown in sulfur-deficient, excess phosphorus medium for 48 h had 7.6-fold more polyP than cells grown in BG-11 medium for the same time (8.4 μg P_i/mg dry wt. as compared to 1.1 μg P_i/mg dry wt.).

Spheroplast formation and analysis allowed further confirmation of the localization of polyP within the cytoplasm. Figure 6 shows the ^{31}P NMR spectra of cells and supernatants during and after spheroplast formation. No polyP was observed in the supernatant after spheroplasts were centrifuged. Only when very high concentrations of polyP were observed in the NMR spectrum of the cells was any polyP peak present in the spectra of periplasmic supernatants. On the other hand, P_i and some sugar phosphates were released upon spheroplast formation (Fig. 6c), as was the case with EDTA treatment alone (Fig. 2). Few cells lysed in this experiment, as shown by only a faint blue color (phycocyanin) in the supernatant analyzed in Fig. 6c. The cytoplasmic contents (Fig. 6d) showed a broad upfield peak of polyP remaining after cell lysis.

Discussion

The presence of polyP in cells of *Synechocystis* sp. strain PCC 6308 was demonstrated by in vivo and spheroplast NMR spectroscopic analysis. It was confirmed by transmission electron microscopic observations and chemical analysis of cells grown under sulfur-stressed, excess phosphate conditions. In vivo ^{31}P NMR spectroscopy of these cells showed a broad peak at –23 ppm that has been identified in other bacteria as polyP (Rao et al. 1985). The broadness of this peak has a number of possible causes. The close proximity of divalent paramagnetic metal ions to negatively charged polyP decreases the relaxation time of the NMR-active nuclei and broadens the peak. In addition, the broad peak results from the overlap of individual peaks from polyP of many different molecular masses. Studies of extracted polyP in *Escherichia coli*, for example, suggest the presence of chain lengths from 13 to over 1,000 phosphate units (Rao et al. 1985).

One goal of this work was to determine the distribution of polyP in the *Synechocystis* sp. PCC 6308 cell. Addition of EDTA or of lysozyme-EDTA to cell suspensions allows the study of polyP distribution. As a prokaryotic cell, a cyanobacterium's only compartments are the cytoplasm and the periplasm. The presence of polyP in the periplasmic space of *Mycobacterium smegmata* has been demonstrated (Ostrovskii et al. 1980). EDTA increases the permeability of the outer membrane of gram-negative bacteria; in *E. coli* the effect is selective, allowing the internal metabolism of the cell to proceed unimpaired (Lieve 1965). It has been suggested that EDTA acts on membranes by initially binding a cation, probably Mg^{2+}, that is associated with lipopolysaccharide and that stabilizes the outer membrane. This binding is followed by a chemical or conformational change in the outer membrane, offsetting the stabilizing effects of the cations (Lieve 1968). EDTA permeabilization has allowed the successful introduction of antibiotics, detergents, and various enzyme substrates of up to mol. mass 1,000 Da into bacteria (Shellman and Pettijohn 1991), suggesting that material up to this size can pass through the permeabilized membrane.

The absence of polyP and the presence of P_i in the NMR spectra of the supernatant of cells treated with EDTA suggest that inorganic phosphate, but not polyP, passes out of the cell under these conditions. It can thus be estimated that the molecular mass of the polyP in the cell is at least 1,000 Da. Inorganic phosphate leaked out of the cell into the medium upon treatment with EDTA, although the cells did not lyse. Lack of lysis was confirmed by finding no water-soluble phycocyanin in supernatants of cells treated with EDTA; phycocyanin readily leaves cells upon lysis. EDTA complexes with cations in the periplasm. There appears to be an equilibrium between cations in the cytoplasm and in the periplasm so that as cations are removed from the periplasm by the EDTA, they are replaced by cations from the cytoplasm. The addition of Mg^{2+} was shown to restore the cells to their normal, nonleaky state, presumably because the excess Mg^{2+} replaced the outer membrane Mg^{2+} that had been previously removed by the EDTA. The added Mg^{2+} also complexed with polyP, causing the rebroadening and change in shift of the polyP NMR signal.

Data presented here suggest that there are two pools of NMR-visible polyP, one with an NMR peak that is shifted and sharpened by the introduction of EDTA, and one that is not. (The large amount of polyP observed by electron microscopy is localized in large, insoluble polyP bodies not visible using NMR spectroscopy.) To eliminate the possibility of a pool of polyP being present in the periplasm, lysozyme-induced spheroplast formation was utilized to follow the release of periplasmic constituents from cells. Only when cells contained a very high concentration of polyP was a small amount of polyP visible in the periplasmic contents. Typically, only a large concentration of P_i was seen in supernatants of pelleted spheroplasts. Thus, the two types of polyP observed in these experiments must be located in the cytoplasm.

If both pools of polyP are in the cytoplasm, why does their behavior differ in the presence of EDTA? The insoluble polyP bodies observed in the electron micrographs consist of heterogeneous polyphosphates with no membrane around them. There may be an equilibrium between the insoluble, NMR-invisible fractions of the polyP bodies and the soluble, NMR-visible ones. There also may be gradients in density and conformation of the soluble polyP, which in turn could affect its capability to bind and release divalent cations. Since the upfield region of the polyP NMR resonance did not narrow or shift in the presence of EDTA, this peak may be associated with a fraction of the soluble polyP that is tightly packed, with most of its divalent cations inaccessible to the EDTA. Another cytoplasmic pool of soluble polyP may be more loosely packed, with its divalent cations accessible to the EDTA. The resonance associated with this polyP thus narrowed and shifted downfield in the presence of increasing con-

centrations of EDTA, up to 10 mM, and was restored by the addition of Mg^{2+}. The constant chemical shift of the polyP between 10 and 20 mM EDTA shown in Fig. 4 suggests either that not all the polyP, even in this pool, is accessible to EDTA, or that all of the divalent cations have been removed by an EDTA concentration of > 10 mM.

In conclusion, in vivo ^{31}P NMR spectroscopy revealed two cytoplasmic pools of cation-complexed, soluble polyP and the insoluble polyP in polyP bodies. The associated cations of only one pool are easily accessible to EDTA. The function and metabolism of the pools are yet to be determined.

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