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Polyphosphate-hydrolysis – a protective mechanism against alkaline stress?

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Different microorganisms, including yeast and algae, accumulate large amounts of polyphosphates. However, the physiological role of polyphosphates is largely unknown. In vivo ^{31}P NMR studies, carried out in the unicellular alga, *Dunaliella salina*, demonstrate that cytoplasmic alkalization induces massive hydrolysis of polyphosphates, which is correlated kinetically with the recovery of cytoplasmic pH. Analysis of acid extracts of the cells indicates that long-chain polyphosphates are hydrolysed mainly to tripolyphosphate. It is suggested that the hydrolysis of polyphosphates provides a pH-stat mechanism to counterbalance alkaline stress.

Polyphosphate; pH regulation; *Dunaliella*

1. INTRODUCTION

Most living microorganisms including bacteria, cyanobacteria, yeast and algae accumulate polyphosphates, which may amount to 10–20% of the dry weight of the cell [1,2]. Since such amounts are far greater than the cellular phosphate requirements, and since the formation of polyphosphates requires a large investment of metabolic energy, it seems conceivable that polyphosphates may have specific functions. However, the role of polyphosphates is still a mystery. It has been suggested that in addition to providing a phosphate reservoir, polyphosphates may serve as an energy reservoir for ATP formation through polyphosphate kinase, which has been identified in bacteria and in yeast [1].

The unicellular halotolerant green alga, *Dunaliella*, accumulates large amounts of polyphosphates equivalent to 0.5–1 M inorganic phosphate, which appear to be associated with K^+ and Mg^{2+} [3,4]. We have recently demonstrated that phosphometabolites in *Dunaliella* can be followed in vivo by ^{31}P NMR spectroscopy performed on cells trapped in agarose beads. In normal cells, the high molecular weight polyphosphates are manifested as a broad hump in the ^{31}P NMR spectrum which probably reflects the restricted motion of packed polyphosphate molecules [5]. Using ^{31}P NMR, the intracellular pH in *Dunaliella* cells has been measured from the position of the intracellular P_i signal, and it was shown to be close to 7 over the extracellular pH range 5–9 [6,7]. It has also been demonstrated that ammonium ions induce cytoplasmic alkalinization in *Dunaliella* cells in the dark, presumably because ammonia diffuses into the cells as NH_3 and is protonated to NH_4^+ inside the cells, thus consuming protons and causing a pH increase [6].

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2. MATERIALS AND METHODS

2.1. *NMR measurements*

D. salina cells were trapped in agarose beads (3% m/v). at a concentration of 6–8 × 10^8 cells/ml, and perfused continously during the NMR experiment (1 ml/min) with a medium containing 0.5 M NaCl, 5 mM $MgCl_2$, and 5 mM KCl, 20 mM Tris-Cl pH 9 saturated with 95% O_2 and 5% CO_2 as described previously [5].

NMR measurements were performed at 12°C in the dark in a Bruker AM-500 NMR spectrometer. ^{31}P spectra were recorded at 202.5 MHz by applying 60° pulses with a repetition time of 1 s. Composite pulse proton decoupling was continuously applied. The spectra were processed using a line broadening of 40 Hz [5].

2.2. *Analysis of polyphosphate breakdown products by thin layer chromatography*

D. salina cells were cultured for 3 days with $^{31}P_i$ (1 mCi/l, a 5 Ci/mol inorganic phosphate) in a 0.5 M NaCl medium in an illuminated incubator as previously described [8]. Concentrated cells (1–2 × 10^8 cell/ml) were extracted either with 14% perchloric acid followed by neutralization of the extract with $KHCO_3$ to precipitate the perchlorate and concentration by lyophilization [9] or with 2 M formic acid (incubation for 30 min at 4°C), followed by lyophilization of the extracts. Samples equivalent to 1–5 × 10^4 cpm were applied to thin layer plates. Cellulose thin layer plates (20 × 20 cm, thickness of 0.25 mm) were developed in the first dimension (vertical) in: Isobutiric acid/NH_3/H_2O/ethylenediaminetetraacetate (250 ml/30 ml/120 ml/0.125 g), and in the second dimension (horizontal) in: *n*-butanol/*n*-propanol/propionic acid/H_2O (200 ml/87.5 ml/142.5 ml/186.5 ml). The plates were dried and exposed to Kodak films for 24 h at 25°C.

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2.3. *Other analytical methods*

Ammonium uptake was measured as follows: *D. salina* cells (2×10^8 cells/ml) were incubated in buffered medium containing 0.5 M NaCl, 5 mM KCl, 5 mM $MgCl_2$ and 20 mM Tris-Cl, pH 9 at 10°C. Following the addition of 20 mM NH_4Cl samples of 200 μl were separated by the silicone oil centrifugation technique [10] and the ammonium content in the cells was determined with the Nessler reagent [11].

ATP content was determined following rapid freezing of cell samples using the luciferase assay in a Lumac 3M Biocounter.

3. RESULTS AND DISCUSSION

Addition of 20 mM NH_4Cl to *Dunaliella* cells at pH 9 induces an immediate arrest of motility and a drop in cellular ATP. However, the cells resume motility and the ATP level is fully restored within 1–2 h (Fig. 1B). In order to find out if the stress is correlated with internal pH changes, cells were trapped in agarose beads, placed in an NMR tube and their intracellular pH was calculated from the shift in the position of the internal P_i peak (Fig. 2).

Introduction of ammonium salt induces a rapid increase (within 5 min), in the intracellular pH from 7.5 to 8.15 which occurs in parallel with a massive accumulation of ammonium inside the cells (Fig. 1A). However, within 2 h the intracellular pH drops back to about 7.8 in spite of the fact that ammonium ions continue to accumulate inside the cells. The ammonium ions also induce a dramatic change in the ^{31}P NMR spectrum which indicates massive hydrolysis of polyphosphates (Fig. 2). The major signals which increase following addition of ammonium ions are in a peak which overlaps the γP of ATP and corresponds to 'terminal phosphates' in polyphosphate chains, and a group of 3 peaks corresponding to 'central phosphates' located in the 2nd, 3rd and 4th positions ($P\text{-}P_2$, $P\text{-}P_3$, $P\text{-}P_4$, respectively). There is a progressive increase in the ratio of 'terminal/'central' phosphates, during the incubation with NH_4^+, indicative of a progressive

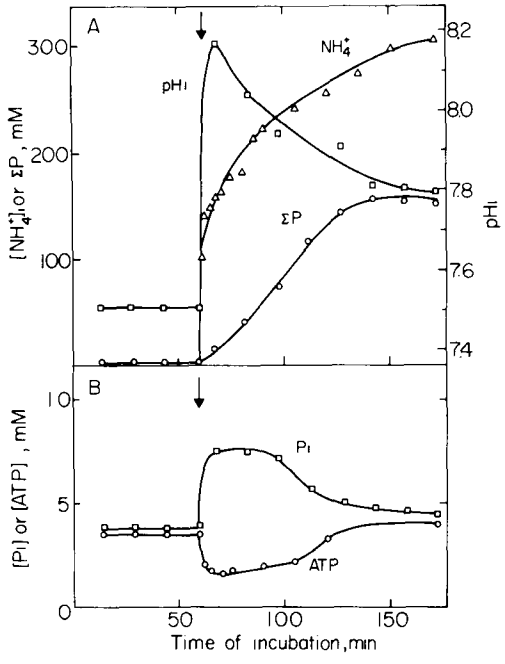

Fig. 1. Correlation between ammonium accumulation, intracellular pH, polyphosphate hydrolysis, ATP and P_i content in *Dunaliella* cells. NMR measurements were performed at 12°C with *D. salina* cells trapped in agarose beads. 20 mM ammonium chloride was added to the perfusion medium at the point indicated by the arrow. Intracellular pH (A) was calculated from the shift in the resonance position of inorganic phosphate (see Fig. 2) by comparison with calibration curves of inorganic phosphate in buffered solutions. Inorganic phosphate concentration (B) and the extent of hydrolysed polyphosphate anhydride bonds (ΣP; ○ in A) were calculated from the areas of the P_i and 'terminal phosphates' signals, respectively (see Fig. 2 and text for further details). Ammonium uptake (Δ in A), and ATP content (○ in B), were measured in a separate experiment under identical conditions as described in section 2.

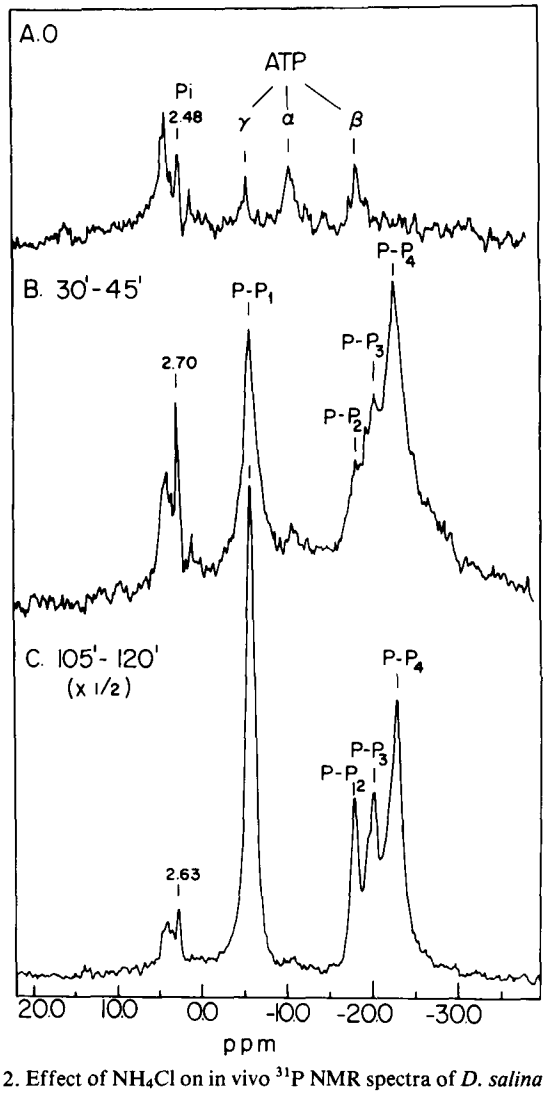

Fig. 2. Effect of NH_4Cl on in vivo ^{31}P NMR spectra of *D. salina* cells. The NMR measurement conditions in *D. salina* cells trapped in agarose beads are described in section 2. Each spectrum represents 900 scan, accumulated over 15 min before (A), 30–45 min after (B), or 105–120 min after (C) administration of 20 mM NH_4Cl. P_i = intracellular inorganic phosphate. The numbers indicate the position of the signal in ppm relative to 85% H_3PO_4. The γP and βP of ATP overlap the 'terminal' ($P\text{-}P_1$) and one of the signals of 'central' phosphates in polyphosphates ($P\text{-}P_2$), respectively.

hydrolysis of polyphosphates (compare Fig. 2C and B). Quantitative estimation of the hydrolysed phosphoanhydride bonds, from the area of the 'terminal phosphate' signal, suggests that over 150 mM equivalent anhydride-bonds have been hydrolysed within 2 h, and that the hydrolysis stops in parallel to the stabilization of the intracellular pH (Fig. 1A). The ammonium ions also induce a smaller increase in intracellular P_i (compare Fig. 2A and B). However the kinetics of the P_i increase differs from that of the polyphosphates: a rapid increase, followed by a decrease within 2 h back to the original level. This transient increase in P_i, resembles the transient decrease in ATP (Fig. 1B) and therefore may indicate that the source of increase in P_i is the hydrolysis of ATP and not polyphosphates.

In an attempt to identify the hydrolytic products of the polyphosphates, *Dunaliella* cells were labeled with ^{32}P, extracted with perchloric or with formic acid and the extracts analysed by thin layer chromatography (TLC). Figure 3 demonstrates that ammonium induces the appearance of several new components. The major one was identified as tripolyphosphate (P-P-P), and the minor components are pyrophosphate (P-P) and tetrapolyphosphate (P-P-P-P). It may be noted that in the perchlorate extraction (Fig. 3A,B) the origin, which presumably contains long chain polyphosphates, is reduced in the ammonium treated cells. Inorganic phosphate is highly intensified in this extraction procedure probably due to partial hydrolysis of polyphosphates. Extraction with formic acid (Fig. 3C) demonstrates the progressive accumulation of tripolyphosphate, the minor accumulation of tetrapolyphosphate and pyrophosphate and the transient drop in ATP. The minor 'origin' observed in Fig. 3C probably reflects a limited extraction of long chain polyphosphates by formic acid. These results are consistent with the ^{31}P NMR experiments and suggest that the ammonium-induced internal alkalinization triggers massive hydrolysis of long-chain polyphosphates to tripolyphosphate.

Interestingly in the yeast, *Saccharomyces cerevisiae*, ammonium ions also induce hydrolysis of long-chain polyphosphates and appearance of tripolyphosphate [12,13]. This phenomenon has been suggested to be associated with nitrogen metabolism.

The following observations are consistent with the suggestion that in the alga, *Dunaliella*, phosphophosphate hydrolysis is induced by the internal alkalinization resulting from influx of ammonia. (i) The time course for recovery of the cytoplasmic pH correlates well with the time course of polyphosphate hydrolysis. (ii) The amount of ammonium taken up by the cells, which roughly equals the number of hydroxyl equivalents produced, is of the same order of magnitude as the amount of hydrolysed phosphate anhydride bonds. It should be noted that if the visibility of the polyphosphate products in the ^{31}P NMR spectrum is incomplete, the true

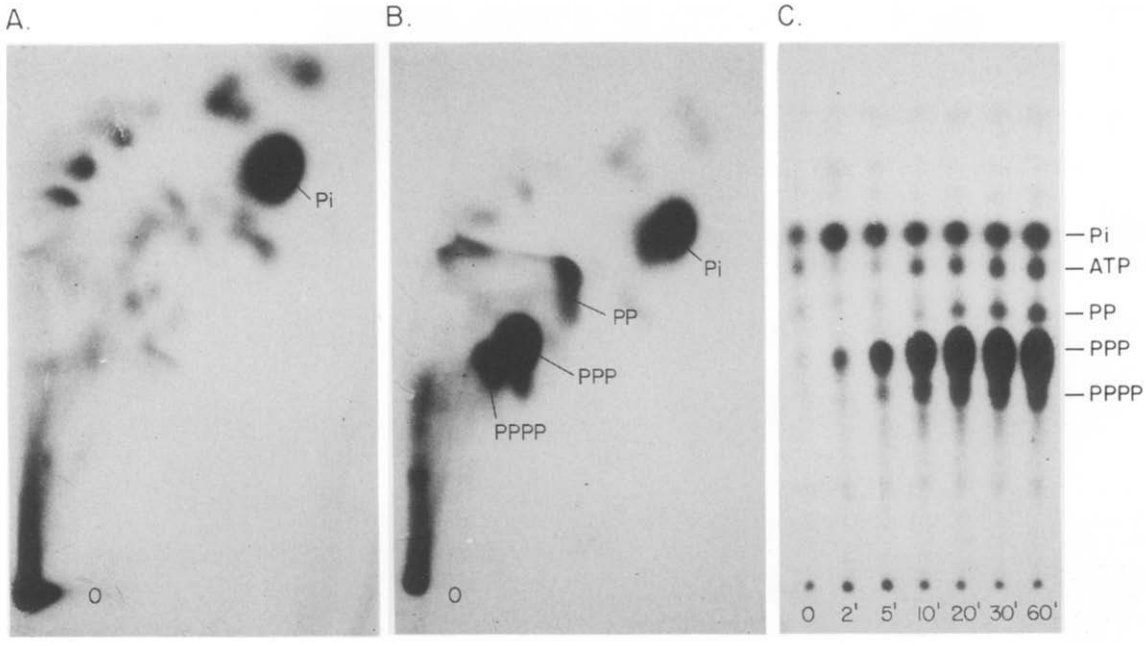

Fig. 3. Analysis of polyphosphate breakdown products in extracts of *D. salina* cells. (A,B)$^{32}P_i$-labeled *D. salina* cells cultured for 3 days were centrifuged and resuspended for 1 h in the absence (A) or presence (B) of 20 mM NH_4Cl. The cells were extracted with 14% perchloric acid, and analyzed on two-dimensional cellulose thin layer plates. (C)^{32}P-labeled *D. salina* cells were incubated with 20 mM NH_4Cl for the indicated time periods, extracted with 2 M formic acid and analysed on cellulose plates which were developed in the solvent composition used for the first dimension in (A,B). Inorganic phosphate (P_i), pyrophosphate (P-P), tripolyphosphate (P-P-P) and tetrapolyphosphate (P-P-P-P) were identified by comparison to unlabeled marker, which were coseparated on the same plates (0.1 μmol/plate) and sprayed with a molybdate reagent [14]. O = origin.

amount of hydrolysed polyphosphate should be even higher. (iii) Other amines, such as benzylamine and triethylamine, which are not metabolized by *Dunaliella* cells, induce a similar cytoplasmic alkalinization as well as hydrolysis of polyphosphates (not shown). These results indicate that the phenomenon is not associated with nitrogen metabolism. (iv) Cells which have been cultured under P_i-deficient conditions, and contain less than 10% of the polyphosphates in control cells, recover slower than control cells from ammonium shock at alkaline pH (not shown).

Taken together, these results suggest that polyphosphates may have a role in counterbalancing alkaline pH stress in *Dunaliella*, and possibly also in other algae, yeast and further microorganisms which accumulate polyphosphates.

Two pathways can be envisaged by which polyphosphate hydrolysis may counterbalance internal alkalinization. Hydrolysis of phosphate anhydride bonds generates protons, as in the case in ATP hydrolysis, and could thus serve as a pH stat. Alternatively polyphosphates may serve as an energy source for ATP formation, which powers an unknown pH-stat mechanism, and that the polyphosphate hydrolysis is part of this reaction. This possiblity is consistent with the transient drop in cellular ATP in response to alkaline stress in *Dunaliella*, and with the presence of polyphosphate kinase activity in several microorganisms which accumulate polyphosphates. At present it is not known whether this enzyme exists in *Dunaliella*.

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