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Polyphosphates as a source of high energy phosphates in yeast mitochondria: a ^{31}P NMR study

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Suspensions of purified yeast mitochondria were analyzed under bubbling oxygen by ^{31}P NMR at 161.9 MHz. The recorded spectra indicate that polyphosphates (poly(P)) are present in mitochondrial preparations. These poly(P) further characterized by the NMR study of mitochondrial perchloric acid extracts have an average chain length of 14 ± 1 residues per chain and correspond to 10% of the total content of cellular poly(P) detected by NMR. The stability of mitochondrial poly(P) was increased by the presence of oligomycin, suggesting that this compound may play a role in the energetic metabolism of yeast mitochondria.

NMR; Polyphosphate; Mitochondria; Energetic metabolism; (Yeast)

1. INTRODUCTION

Polyphosphates (poly(P)) are common in many organisms encompassing bacteria and mammals [1]. In *Saccharomyces cerevisiae*, the poly(P) account for about 37% of the total phosphate content and consist of linear chains with 20 phosphates units or even more [2]. Their subcellular localization has been studied by several authors, the results indicate that the major part of the poly(P) of *S. cerevisiae* is localized in cytoplasmic vacuoles [3]. A limited amount has been found associated with the outside plasma membrane, which represents about 10% of the total cellular content [4] and in the nucleus of the cell.

The biological function of poly(P) is still a matter of dispute; however, a role in the regulation of metabolic processes was well established [1,5]. The synthesis of poly(P) is mainly realized by the polyphosphate kinase which catalyses the reaction:

$$ATP + poly(P)n \rightleftharpoons ADP + poly(P)n + 1$$

The reverse reaction, i.e. the synthesis of ATP from poly(P), is favorized through a processive mechanism, then poly(P) may also serve as storage form of energy, as a direct ADP-phosphorylating agent.

^{31}P NMR has been used to investigate the metabolism of phosphorylated compounds in wild-type and mutants of *S. cerevisiae* [6]. Studies on yeast mitochondria have not been reported but extensive NMR work has been done by Ogawa et al. on rat liver mitochondria [7,8]. Here, we present an NMR investigation on mitochondria purified from yeast cells. The main result is the occurrence in the ^{31}P NMR spectrum of resonances which can be assigned to poly(P). A direct consequence of this finding is that poly(P) can play a role in the energetic metabolism of mitochondria.

2. MATERIALS AND METHODS

2.1. *Yeast strain, media and growth*

The diploid yeast strain used is the wild-strain *Saccharomyces*

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid

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cerevisiae (yeast foam). Cells were grown aerobically at 28°C on a medium at pH 4.5, containing 1% yeast extract, 0.1% potassium phosphate, 0.12% ammonium sulfate and supplemented with 2% lactate as the carbon source. Cells were harvested in the logarithmic growth phase.

2.2. *Preparation and purification of yeast mitochondria*

Mitochondria were isolated from protoplasts as already described [9]. Protein concentration was measured by the biuret method using bovine serum albumin as standard. When required, mitochondria were loaded onto a discontinuous sucrose gradient (55%, 40% and 30%, w/v, sucrose, respectively) in 10 mM Tris-maleate buffer, pH 6.8. After centrifugation at 135000 × *g* for 15 min in a Beckman TL-100 ultracentrifuge, the purified mitochondria were recovered from the interface between the 40 and 55% sucrose layers [10].

The cytochrome ($a+a_3$) content of cells and isolated mitochondria was measured at room temperature with a Perkin-Elmer 356 spectrophotometer. Since cytochrome ($a+a_3$) is an accurate marker of mitochondria, we can estimate the fraction of mitochondrial proteins per mg cell dry wt and then the mitochondrial poly(P) pool in the cell.

2.3. *Perchloric acid extracts*

Mitochondria (50–60 mg of proteins) were acid extracted with 7% (w/v) $HClO_4$ at 4°C. The precipitated proteins were removed by centrifugation at 4°C. The supernatant was adjusted to pH 6.0 with 2 M KOH/0.3 M Mops and the resulting pellet was removed by centrifugation at 4°C. The extract was lyophilized prior to NMR measurements.

2.4. *NMR spectroscopy*

In vivo ^{31}P NMR spectra were recorded at 161.9 MHz using a Bruker AM400 spectrometer. Spectra were obtained at 25°C with 20 mm NMR tubes containing 10 ml of cells or mitochondria suspensions, continuously bubbled with 95% O_2, 5% CO_2. Acquisition parameters were the following: 60° pulse angle, 0.135 s acquisition time, 0.065 s (for entire cells) or 0.165 s (for mitochondria) of delay, 1500 scans and 4K memory size. The cells (500 mg dry wt) were suspended in a buffer containing 2 mM $MgSO_4$, 1.7 mM NaCl, 2 mM potassium phosphate, 50 mM Mes and 2% lactate as the carbon source [11]. The pH was adjusted to 6.0 using NaOH. The mitochondria (120–170 mg of proteins) were suspended in the following medium: 0.65 M mannitol, 0.36 mM EGTA and 25 mM Pipes at pH 6.7.

For NMR analysis of perchloric acid extracts, 3 ml of D_2O containing the lyophilized material (corresponding to around 50 mg of proteins) and 25 mM EDTA were placed in a 10 mm NMR tube. Spectra were obtained overnight by using a 45° pulse angle, 0.41 s acquisition time, 0.1 s of delay and 120000 scans (memory size 8K). Saturation parameters for *T*1 relaxation corrections were obtained by recording one spectrum under fully relaxed conditions (10 s of delay).

Chemical shifts were given by using an internal reference of methylenediphosphonic acid at 18.6 ppm. Peak areas were determined either by using a software integration routine or by cutting and weighing the peaks and by comparing their area with that of the methylenediphosphonate standard.

3. RESULTS

The ^{31}P NMR spectrum of a cellular suspension of yeast under aeration is shown in fig.1A. This spectrum is similar to those already published [6,12] and the resonances can be assigned from previous papers as well as from perchloric acid extract spectra (not shown). Peak assignments are given in the legend of fig.1. From the position in the frequency scale of the resonances belonging from inorganic phosphate, the intracellular pH (pHin) can be estimated to 7.20 ± 0.05 and the external pH (pHex) to 6.05 ± 0.05. Fig.1B shows the spectrum recorded from a suspension of yeast mitochondria: the most interesting feature of the spectrum is the presence in the preparation of a large amount of poly(P), as illustrated by the signal at −22.5 ppm (PP4, PP5) corresponding to the inner phosphates of poly(P). Since a large part of the poly(P) found in yeast cells are localized within cytoplasmic vacuoles, it can be possible that the poly(P) resonances appearing in spectrum 1B come from vacuolar poly(P).

In order to document better the origin of the poly(P) pool, the suspension of mitochondria was further purified by centrifugation through a sucrose gradient, as indicated in section 2. The ^{31}P spectrum recorded from freshly purified mitochondria, in the presence of O_2 and oligomycin is given in fig.2A. It is very similar to the spectrum shown in fig.1B and these data indicate that poly(P) are tightly associated to or within the yeast mitochondria. To help with the identification of the peaks, we made a perchloric acid extract of mitochondria, shortly after the centrifugation step. Fig.2B shows the entire spectrum of the extract while expanded plots of this spectrum are shown in fig.3. In the high-field region, the resonances come mainly from inner phosphate of long chain poly(P) at −22.55 ppm (PP5), the inner phosphate of triphosphate centered at −22.29 ppm (PP2 in poly(P)$_3$, triplet), the β-phosphate of nucleoside triphosphates and the PP3 centered at −21.95 ppm (β-NTP and PP3, triplets) and the penultimate phosphates of poly(P) between −21.5 ppm and −22.5 ppm (PP2) [6]. Other resonances coming from poly(P) are visible in the −6.5 ppm to −9 ppm region: in particular the PP1 signal around −7 ppm (phosphate end group); the doublet centered at −8.25 ppm and

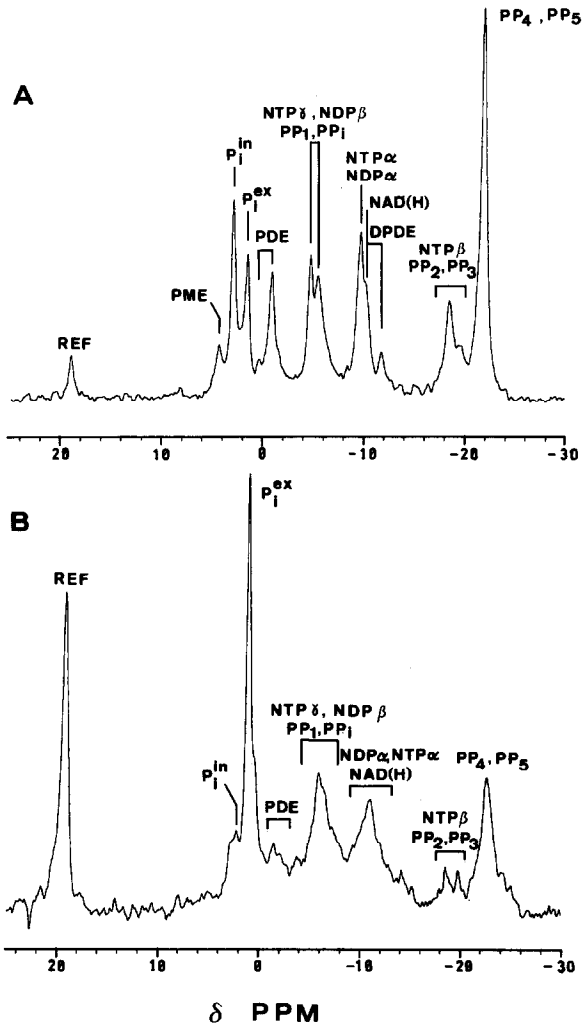

Fig.1. ^{31}P NMR spectra of suspension of yeast cells and yeast mitochondria. Spectra were recorded as described in section 2, (A) whole cells (50 mg/ml dry wt), 1500 scans, 5 min accumulation; (B) crude mitochondrial fraction (17 mg of proteins/ml), 1500 scans, 7.5 min accumulation. P_i^{in} and P_i^{ex}, intracellular and external inorganic orthophosphate, respectively; PME, phosphomonoesters; PDE, phosphodiesters (mainly GPC, glycerophosphocholine and GPE, glycerophosphoethanolamine); NTP and NDP, nucleoside tri- and diphosphates; DPDE, diphosphodiesters (mainly UDPG); PP_i, inorganic pyrophosphate; PP_n, phosphate residue at the nth position in polyphosphate chains; REF, reference signal of methylenediphosphonic acid (capillary).

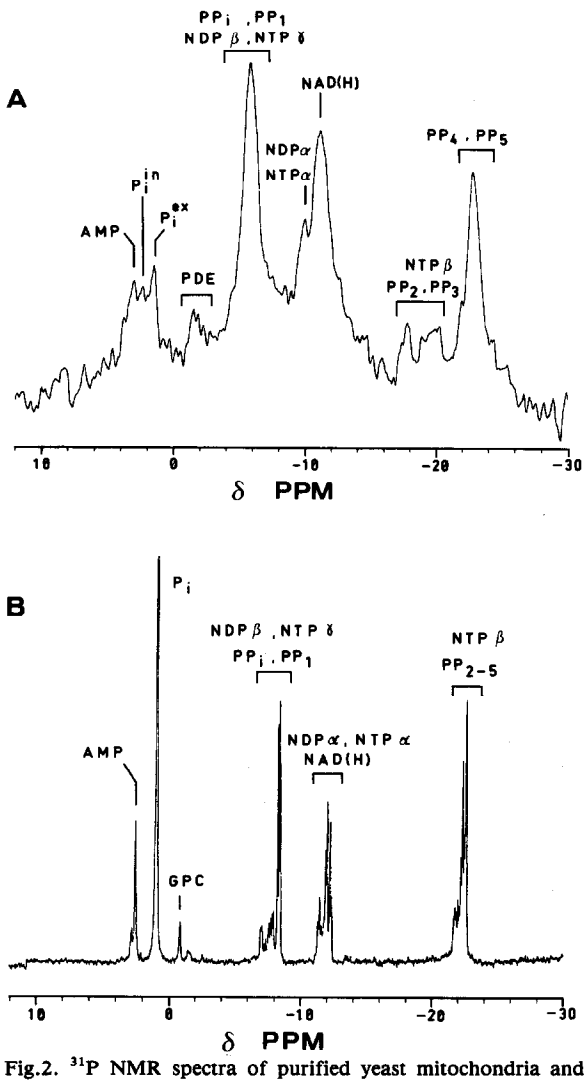

Fig.2. ^{31}P NMR spectra of purified yeast mitochondria and mitochondrial perchloric acid extract. (A) Spectrum recorded from a suspension of mitochondria (12 mg of proteins/ml) in the presence of oligomycin (20 μg/mg of proteins), 1500 scans, 7.5 min accumulation. (B) Perchloric acid extract (50 mg of proteins). Abbreviations as in fig.1.

the singlet at −9.45 ppm belong to the external phosphate of poly(P)3 and PP_i, respectively.

From the extract spectrum, it is also possible to estimate the average chain length of the poly(P), by using the relative intensities of the signals of inner, penultimate and end phosphates of poly(P) [6]. A ratio of 7 (after correction for saturation) for the PP5 peak relative to the penultimate phosphate signal was calculated corresponding to an average length of 14 ± 1 residues per chain.

The stability of the mitochondrial poly(P) pool has been followed noninvasively by recording sequential NMR spectra from a suspension of purified mitochondria: a rapid poly(P) hydrolysis was observed (6 ± 1 min half time) under our ex-

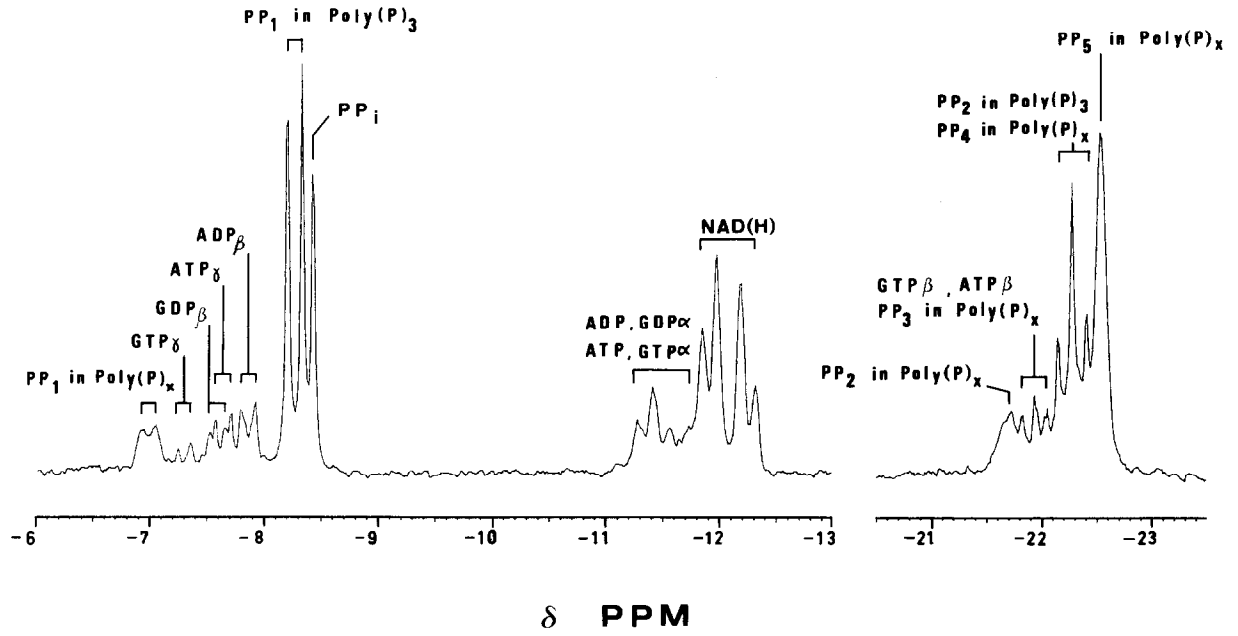

Fig.3. Expanded regions of the ^{31}P NMR spectrum of perchloric acid extract of yeast mitochondria. Same conditions and abbreviations as in fig.2. PP_n is the phosphate residue at the nth position; $poly(P)_x$, polyphosphate of x residues chain length.

perimental conditions, correlated with an increase in external P_i. In the presence of oligomycin (20 μg/mg of proteins), an inhibitor of mitochondrial ATPase activity, the ATP pool is maintained at basal level, then the rate of poly(P) hydrolysis is significantly reduced (more than a factor 2), suggesting that poly(P) may contribute to ATP synthesis (results not shown).

4. DISCUSSION

In yeast, poly(P) can reach a chain length of more than 260 phosphate units [13]. However, more recently, it has become clear that a specific fraction of poly(P) represents a pool of poly(P) localized in various compartments of the cell [14]; very high molecular mass poly(P) seems to be located at the cell periphery, whereas short chain poly(P) from 4 to 20 residues, were found, at least partly, in the cytoplasm and the nucleus [14]. ^{31}P NMR allows the detection of soluble poly(P), a part of them being located on the exterior of the whole cell surface [15]. The data presented here indicate that poly(P) from 4 to 15 phosphate residues are also present in purified mitochondrial preparations; this mitochondrial pool can be estimated to be 10% of the total NMR-detected poly(P).

To our knowledge, the presence of a polyphosphatase activity in mitochondria has never been described; the mitochondrial membrane-bound pyrophosphatase is active only with pyro- and triphosphate as substrates (also present in mitochondria). A poly(P) kinase has been purified from *S. cerevisiae* by Felter and Stalh [16]. The enzyme was active in phosphorylating ADP with poly(P) and much less active in the reverse direction. If such an enzymatic activity is present in the mitochondria, then mitochondrial poly(P) could act as a 'phosphagen' molecule.

In this work, experimental evidence for the contribution of poly(P) in mitochondrial energetic metabolism has been obtained. Mitochondrial ATP generation directly from poly(P) might represent a new pathway that we intend to examine to gain a better understanding of mitochondrial energetic metabolism.

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