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^{31}P NUCLEAR MAGNETIC RESONANCE ANALYSIS OF PENICILLIUM OCHRO-CHLORON

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^{31}P nuclear magnetic resonance (NMR) spectra of a filamentous fungus P. ochro-chloron were obtained at a frequency of 40.3 MHz. The spectra show resonances assignable to sugar phosphates, orthophosphates, glycerol-3-phosphorylethanolamine (GPE), glycerol-3-phosphorylcholine (GPC), ATP-Pα, UDPG and polyphosphate. The intracellular pH is estimated to be 6.4 in the logarithmic phase of growth, and high levels of GPE and GPC are characteristic of the fungus. The ^{31}P NMR spectra reflect the age of the fungus and show the accumulation of polyphosphate in the stationary phase.

INTRODUCTION

^{31}P nuclear magnetic resonance (NMR) has become a widely used technique for the nondestructive analysis of the quantitative profile and of the chemical environment of many biological phosphorus compounds in vivo. This methodology has been successfully applied to various cellular systems ranging from bacteria to mammalian cells (1). The application of ^{31}P NMR to filamentous fungi, however, has apparently not yet been reported.

In this report, we show that ^{31}P NMR can be used to detect phosphate metabolites in a heavy metal tolerant filamentous fungus, Penicillium ochro-chloron (2), and to characterize its intracellular phosphorus compounds in vivo. Also presented is a comparison between the ^{31}P NMR spectra in the logarithmic phase and stationary phase of growth.

MATERIALS AND METHODS

Culture condition: Penicillium ochro-chloron, strain ATCC 36741, was grown in 500 ml Erlenmeyer flasks containing 150 ml of the chemically defined medium (2) which contained 0.25% KH_2PO_4 as a sole phosphorus source at 30°C on a rotary shaker. After growth to the logarithmic phase (3 days) and to the stationary phase (6 days), the mycelia were harvested on a suction filter.

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Intact cell sample: The mycelium harvested was washed three times with cold 50 mM NaCl, 5 mM Tris-HCl buffer, pH 7.4, packed into a 10 mm diameter NMR tube and analyzed by ^{31}P NMR immediately.

$HClO_4$ extract sample: The mycelium harvested was washed three times with cold deionized water and extracted with cold 6% $HClO_4$ followed by sonication at 4°C for 15 min. After centrifuging, the supernatant was neutralized with cold 5 M KOH, and $KClO_4$ precipitate was removed by centrifugation at 4°C. The $HClO_4$ extract was lyophilized and dissolved in a small amount of deionized water. EDTA was added to 3% (w/v) and the pH was adjusted with 1 M HCl or NaOH.

NMR measurement: ^{31}P NMR spectra were obtained at 40.3 MHz on a JEOL JNM-FX 100 spectrometer operating in the Fourier transform mode. The spectra were recorded with proton noise decoupling and sample spinning. A 3 mm capillary which contains 2% methylenediphosphonic acid (MDP) in D_2O was inserted coaxially into the 10 mm NMR tube. Field homogeneity was adjusted and the field locked on the deuterium resonance of external D_2O in the capillary, and chemical shifts were referenced to external MDP in the capillary at -18.18 ppm relative to 85% H_3PO_4 at 0 ppm.

Nucleotide analysis: The nucleotides in the $HClO_4$ extracts were separated by high-performance liquid chromatography (HPLC) as described by Anderson et al. (3). HPLC was performed with a JASCO TRI ROTAR-III liquid chromatograph equipped with a JASCO UVIDEC-100-III UV detector at 260 nm. A JASCO Finepak SIL C_{18} column (4.6 mm x 250 mm) was used at 40°C.

RESULTS AND DISCUSSION

Fig. 1(A) shows the ^{31}P NMR spectrum of P. ochro-chloron intact cells in the logarithmic phase. The cells are thought to be resting because the mycelium was packed in the washing solution at low temperature throughout the NMR measurement. The spectrum was the sum of 2244 free induction decays at intervals of 1.5 sec. and total accumulation time was 56 min. Several broad peaks were observed in the range from -3 to 23 ppm. The resonances were assigned on the basis of their chemical shifts, and from chemical shifts, spin coupling and pH titration of signals in the $HClO_4$ extract spectrum shown in Fig. 1(B).

The weak peak A at -2.5 ppm is due to sugar phosphates corresponding to the three peaks at -4.47, -4.11 and -3.92 ppm in the $HClO_4$ extract spectrum. The peak B at -1.1 ppm is assigned to intracellular orthophosphate (Pi^{in}). From the chemical shift of the Pi^{in} peak, the intracellular pH is estimated to be 6.4 using a titration curve of 10 mM potassium phosphate in the presence of 100 mM KCl (4). The intracellular pH value of P. ochro-chloron is almost identical with that of a yeast Saccharomyces cerevisiae in the resting state (5).

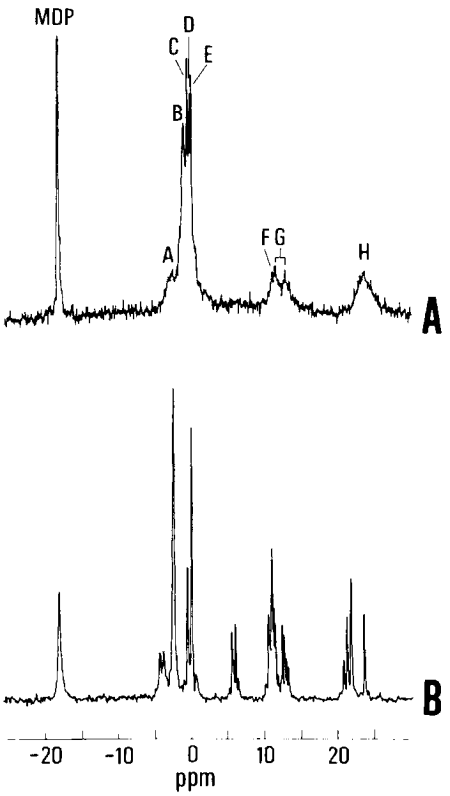

Figure 1. ^{31}P NMR spectra of P. ochro-chloron in the logarithmic phase: (A) intact cells, (B) $HClO_4$ extract (pH 7.4). The chemical shifts are referenced to external 2% methylenediphosphonic acid (MDP) in D_2O at -18.18 ppm. Spectrometer conditions: spectral width 3 kHz, 8 k data points, pulse width (A) 13 µsec (90°), (B) 7 µsec (45°), 1.5 sec repetition time, accumulation (A) 2244, (B) 4881, 4°C.

The peak D at -0.2 ppm is assumed to be another orthophosphate, since similar to the Pi^{in} peak B, the peak D seems to correspond to the Pi resonance at -2.54 ppm in the $HClO_4$ extract spectrum and when the intact cells spectrum was measured without proton noise decoupling the intensity of the peak D did not reduce (6). The chemical shift of the peak D corresponds to extremely acidic pH (below 5). Hence, the tentative assignment of the peak D is to external Pi considering that the pH of the medium, initially 4.0, decreased to ca. 2 in the logarithmic phase.

The intense resonances C at -0.6 ppm and E at 0.0 ppm are assigned to glycerol-3-phosphorylethanolamine (GPE) and glycerol-3-phosphorylcholine (GPC), respectively (7). Relatively high levels of GPE and GPC are characteristic of P. ochro-chloron. GPE and GPC are hydrolysates of predominant

phospholipids in P. ochro-chloron, phosphatidylethanolamine and phosphatidylcholine, respectively. To our knowledge, phospholipase B which catalyzes hydrolysis of phospholipids has been isolated only from Penicillium notatum (8). Therefore, it is suggested that the hydrolysis of phospholipids with phospholipase B and the accumulation of GPE and GPC are specific metabolism in Penicillium species.

The peaks F at 10.9 ppm and G at 11.3 and 12.7 ppm are attributed to ATP-Pα and UDPG, respectively. The assignments were confirmed by nucleotide analysis of the $HClO_4$ extract with HPLC. In the chromatogram, peaks of UDPG and ATP were mainly observed, but a NAD^+ peak was not detected. The resonances of ATP-Pβ and -Pγ which appeared in the $HClO_4$ extract spectrum centered at 21.13 and 5.72 ppm, respectively, were not observed in the intact cells spectrum, probably owing to broadening by the low intracellular pH and/or by binding of divalent metals.

Finally, the peak H at 23.3 ppm comes from central phosphate groups in inorganic polyphosphate. Polyphosphate is widely distributed among the bacteria, fungi and algae, and the physiological role is supposed to be phosphate storage, but not elucidated well (9). The resonance of the polyphosphate central groups in the intact cells spectrum is shielded by 2 ppm relative to that in the $HClO_4$ extract spectrum at 21.47 ppm. Glonek et al. reported that in aqueous solution in the presence of Na^+ ion the chain polyphosphate assumes a helical conformation with each turn of the helix corresponding to three PO_4 tetrahedra, whereas in the presence of the helix disturbing agents such as tetra-n-butylammonium ion in water or tetramethylurea in anhydrous condition the ^{31}P resonance of polyphosphate is shifted upfield by 2-3 ppm relative to its position in water in the presence of Na^+ ion (10). The upfield shift of the polyphosphate peak in the intact cells spectrum might indicate that the polyphosphate in the P. ochro-chloron cells occurs in somewhat disturbed helix conformation.

In the ^{31}P NMR spectrum of the $HClO_4$ extract, a pH independent signal resonated at 23.40 ppm which has never reported in biological samples was

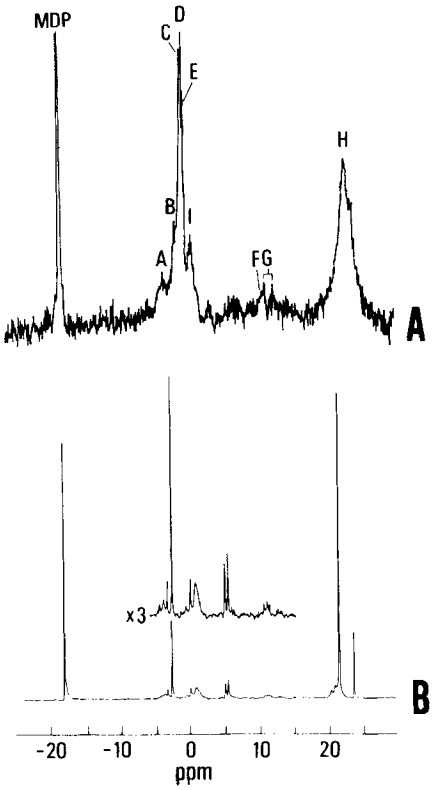

Figure 2. ^{31}P NMR spectra of P. ochro-chloron in the stationary phase: (A) intact cells, (B) $HClO_4$ extract (pH 7.4). Spectrometer conditions were the same as in Figure 1 except for accumulation (A) 2009, (B) 3314.

seen upfield by 2 ppm from the polyphosphate peak at 21.47 ppm. The resonance remains unidentified, but is most likely to be tetrametaphosphate as judged from its chemical shift (10).

The ^{31}P NMR spectra reflect the age of the fungus. Fig. 2 shows the ^{31}P NMR spectra of P. ochro-chloron in the stationary phase: (A) intact cells, (B) $HClO_4$ extract. Compared with the spectra in the logarithmic phase, the intensity of the polyphosphate peak increased remarkably, showing the accumulation of polyphosphate in the stationary phase. This result is consistent with the known fact that polyphosphate accumulates when a organism is placed in conditions unfavourable to growth (9). The peaks arised from terminal phosphate of the polyphosphate was observed at 4.99 and 5.43 ppm in the $HClO_4$ extract spectrum in the stationary phase (11). The Pi^{in} peak B at -1.4 ppm shows the intracellular pH is 6.7. A new peak I appeared at 0.9 ppm in the

stationary phase, which was also observed at 0.74 ppm with broad linewidth in the $HClO_4$ extract spectrum. The resonance occurring in the phosphodiester region such as nucleic acids is not identified and the assignment of the resonance is under investigation.

It is clear from these findings that ^{31}P NMR can be a useful means of both the characterization and metabolic study of cellular phosphorus containing substances in filamentous fungi in vivo.

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