³¹P Nuclear Magnetic Resonance Study of Growth and Dimorphic Transition in *Candida albicans*

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A ³¹P NMR study of the fungal pathogen *Candida albicans* was carried out. Yeast-form cells at different phases of growth, as well as germ tubes and hyphae were examined. In all cases, the NMR spectra showed well separated resonance peaks arising from phosphorus-containing metabolites, the most prominent being attributable to inorganic phosphate (P_i) polyphosphates, sugar phosphates and mononucleotides, NAD, ADP and ATP. Relevant signals were also detected in the phosphodiester region. The intensity of most signals, as measured relative to that of P_i, was clearly modulated both at the different phases of growth and during yeast-to-mycelium conversion, suggesting significant changes in the intracellular concentration of the corresponding metabolites. In particular, the intensity of the polyphosphate signal was high in exponentially growing, yeast-form cells, then progressively declined in the stationary phase, was very low in germ tubes and, finally, undetectable in hyphae.

NMR spectral analysis of the P_i region showed that from early-stationary phase, P_i was present in two different cellular compartments, probably corresponding to the cytoplasm and the vacuole. From the chemical shift of P_i, the pH values of these two compartments could be evaluated. The cytoplasmic pH was generally slightly lower than neutrality (6.7–6.8), whereas the vacuolar pH was always markedly more acidic.

INTRODUCTION

High resolution NMR spectroscopy has recently been applied to the study of intact living systems in a variety of normal and pathological conditions (Shulman *et al.*, 1979; Radda & Seeley, 1979; Gadian & Radda, 1981; Gadian, 1982). A distinct advantage of this technique is the possibility of gaining detailed information on the level of several metabolites, as well as on their interconversion *in vivo* under non-disruptive, non-invasive conditions. This represents an ideal complement and/or alternative to classical biochemical and cytochemical studies.

Previous studies have shown that intracellular pH can be measured by ³¹P NMR spectroscopy (Moon & Richards, 1973; Burt *et al.*, 1979) and the level of phosphate and of phosphorus-containing metabolites monitored in many cellular systems (Hollis, 1980; Radda & Seeley, 1979; Shulman *et al.*, 1979), including the yeast *Saccharomyces cerevisiae* (Salhany *et al.*, 1975; Navon *et al.*, 1979). To our knowledge, no ³¹P NMR study of the dimorphic fungus *Candida albicans* has been reported. In the present investigation, we examined the feasibility of the ³¹P NMR approach for examining the dimorphic transition, i.e. the conversion from a yeast

Abbreviation: PCA, perchloric acid.

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to a mycelial habit of growth which is so characteristic of, and important in, this organism. This transition is accompanied by marked modifications in cell-wall chemistry and ultrastructure (Chattaway *et al.*, 1968; Cassone *et al.*, 1973), in the plasma membrane and general metabolism (Marriott, 1975; Chattaway *et al.*, 1973; Land *et al.*, 1975) as well as by an increase in virulence of the microorganism (Odds, 1979). The exact nature of the factor(s) which controls the transition is, however, completely unknown. This paper reports the first results of ^{31}P NMR studies of *C. albicans* and describes modulations in the concentration of important phosphorylated metabolites, the polyphosphate/phosphate ratio and, the measurement of the intracellular pH during growth in the yeast or mycelial form.

METHODS

Organism and growth conditions. *Candida albicans* strain BP, which was used for this study was isolated from a clinical specimen and identified according to the established taxonomic procedure (Lodder, 1970). It was routinely maintained on Sabouraud agar slopes, at 28 °C. For growth in the yeast form, the organism was transferred to a low-glucose, Winge medium (Mattia & Cassone, 1979) and incubated under moderate agitation at 28 °C. When required, the medium was supplemented with Na_2HPO_4 at a final concentration of 20 mM. At different time intervals, corresponding to the different phases of growth, samples of culture were removed, washed three times in distilled water and packed into the NMR tube. For growth in the mycelial form, early (24 h)-stationary phase cells were washed and incubated in *N*-acetylglucosamine medium, at 37 °C (Simonetti *et al.*, 1974). At 90 min and 240 min (when germ tubes and hyphae, respectively, were formed), the cultures were harvested on Millipore filters (type HA: Millipore) and the organisms washed and packed into the NMR tube. In all cases, a similar amount of fungal mass, corresponding to about 5×10^{11} yeast cells was packed in D_2O (20%, v/v), in a final volume of 2 ml. In some experiments, inorganic phosphate (K_2HPO_4 , at a final concentration of 12.5 mM) was added to packed cells. The external pH of all samples examined by NMR was roughly the same and did not extensively change during measurements. Cell extracts for ^{31}P NMR analysis were made by freezing and thawing cells suspended in 30% (w/v) perchloric acid, essentially as described by Navon *et al.* (1979).

NMR measurements. ^{31}P NMR spectra of *C. albicans* at the distinct phases of growth were recorded at 4 °C by means of a Varian FTXL 100-15" spectrometer working at 40.5 MHz, interfaced to a 620-L computer, under conditions of broad-band proton decoupling. The field/frequency ratio of the spectrometer was stabilized by 'locking' on the D_2O resonance. The sample temperature was controlled to ± 1 °C with a Varian V4340 temperature control unit. Sample tubes of 12 mm (outside diameter) were used. Each spectrum represented the Fourier transform of accumulated free induction decays, obtained with a sequence of 60° pulses and acquisition time of 1.00 s for the cells and 1.50 s for the extracts.

^{31}P NMR spectra of the cells at various stages of the dimorphic transitions were carried out at 80-95 MHz and 4 °C in 10 mm (outside diameter) sample tubes using a Bruker WP-200 superconducting spectrometer interfaced to a computer ASPECT 2000. Repetitive pulses of 60° and an acquisition time of 1.64 s were used. The frequency of resonance signals (chemical shift) was measured in p.p.m. relative to the 85% (w/v) phosphoric acid (external reference) contained in a capillary tube inserted coaxially into the cell sample tube.

All spectra were generally taken within 2 h of harvesting the organism. When longer accumulation times were used (up to 10 h), no significant spectral variations were observed in line width and relative peak area. The intracellular pH value(s) was measured from the chemical shift of the inorganic phosphate signal(s) using 5 mM-potassium phosphate as a standard (Roberts *et al.*, 1981). No corrections were made to the calculations of peak areas to take into account different saturation and Nuclear Overhauser Effect Enhancement factors of the various compounds. Therefore, no absolute concentrations of the assigned compounds are reported and only their relative variations during growth and mycelial conversion were considered, when indicated. In particular, the intensities of the various signals were always taken relative to that of the intracytoplasmic inorganic phosphate.

RESULTS

^{31}P NMR spectra of intact cells of *C. albicans* at different phases of growth in the yeast form

^{31}P NMR spectra characteristic of different phases of growth were reproducibly obtained (Fig. 1). An overall view of these spectra showed the following main resonances. (1) A rather broad band (I) resulting from a number of partially overlapping peaks and centered at $\sim +3$ p.p.m. downfield from the signal of the external reference (see Methods). This band contains resonances of sugar phosphates, including the glycolytic intermediates, as well as of mononucleotides. (2) Two signals (II and III) centered at $\sim +1.2$ and $\sim +0.3$ p.p.m., respectively (in 48 h stationary phase cells), both attributable to intracellular P_i . (3) A narrow

Fig. 1

Fig. 1. ^{31}P NMR spectra at 40.5 MHz, 4 °C, of *C. albicans* at different phases of growth in the yeast form. (a) Exponential phase (8 h); (b) stationary phase (40 h); (c) stationary phase (48 h); (d) stationary phase (48 h) after growth in Wingate medium with an additional 20 mM- Na_2HPO_4 . For other details, see Methods.

Fig. 2

Fig. 2. ^{31}P NMR spectra at 40.5 MHz, 30 °C, of PCA-extracts of: (a) stationary phase (42 h), yeast-form cells; (b) hyphae (240 min in *N*-acetylglucosamine medium). Both extracts were at pH 7.85. For other details, see Methods.

signal (IV) resonating at ~ -0.2 p.p.m. (4) A broad composite band (V) centered at ~ -1.8 p.p.m. in a spectral position typical of compounds with phosphodiester bonds. (5) A very prominent resonance (IX) centered at ~ -23 p.p.m. and attributed to polyphosphates. (6) In the frequency interval between signals V–IX, distinct, complex resonance bands were detected. Their spectral positions correspond to those typical of γ -ATP and β -ADP (VI), α -ATP, α -ADP and NAD (VII), and β -ATP (VIII).

Resonances II and III can be attributed to P_i localized within two distinct cellular compartments, at different pH values. No such clear compartmentation was seen in exponential phase yeasts, which essentially showed only one resonance at the position of signal II (Fig. 1a). The intensity of signal III became significant in yeasts after 36–48 h of growth (Fig. 1b, c).

The intensities of signals IV and V were significantly modulated during growth in the yeast form. The intensity of signal IV apparently increased in stationary phase cells as compared with exponentially growing cells, whereas the intensity of signal V was lowest in the late-stationary phase (Fig. 1a–c). Analogous changes were seen in the spectra of PCA-extracts of cells at different phases of growth. In particular, signal V was absent in the extracts of cells from late-stationary phase (Fig. 2a).

The ratio between the intensities of the polyphosphate and P_i (II + III) signals showed a marked decrease during growth: it measured about 2.7 in exponentially growing cells (Fig. 1a), 1.4 after 24 h of growth (early-stationary phase, see below; Fig. 3a), 0.8 at 40 h (Fig. 1b) and 0.3

Fig. 3. ^{31}P NMR spectra at 80-95 MHz, 4 °C, of *C. albicans* in different forms of growth: (a) yeast-form (early-stationary phase, 24 h); (b) germ tubes; (c) hyphae. For other details, see Methods.

in late-stationary phase cells (48 h; Fig. 1c). Moreover, the polyphosphate signal shifted slightly downfield with cells progressing in the stationary phase (Fig. 1a-c). Growth in medium containing an additional amount of inorganic phosphate (see Methods) brought about a significant increase in the relative polyphosphate content as well as in the intensity of signal III (measured in the late-stationary phase of growth; Fig. 1d).

Modulation of ^{31}P NMR signals during yeast to mycelial transition

In an attempt to gain some insight into the biochemical changes which occur during yeast-to-mycelium transition, early (24 h)-stationary phase yeasts, germ tubes and hyphae were examined by ^{31}P NMR. Apart from changes in chemical shift (and in the corresponding pH values) of signals II and III (see below), other significant modulations were observed in the spectra of the different forms of growth (Fig. 3). The intensity of signal IV decreased in germ tubes as compared with yeasts and was no longer appreciable in the hyphae (although it was detectable in the concentrated hyphal PCA-extract (see Figs 2b and 3a-c). On the other hand, signal V was found both in whole organisms in the different forms of growth and in the PCA-extracts, with the exception of the extract obtained from stationary phase cells where it was absent (Figs 3a-c, 2a). During yeast-to-mycelium conversion, no appreciable changes were found in the relative intensity of the sugar-phosphate band (I) while ATP and ADP signals showed a lower intensity in the germ tubes and hyphae as compared with yeast cells.

A comparison of the spectra reported in Fig. 3 shows that the polyphosphate signal, which was conspicuous in the yeast form, was greatly reduced in the germ tubes and disappeared in the hyphal form. In particular, the ratio between polyphosphate and P_i signal areas fell from 1.4 in yeast-form cells to 0.5 in germ tubes and to zero in hyphae.

Phosphate compartmentation and pH values of the cytoplasm and the vacuole in the distinct phases and forms of growth

As shown before, two signals (II and III) arose from P_i localized in two different cellular compartments assumed to be the cytoplasm and the vacuole, respectively (see Discussion). Table 1 summarizes the pH values of these two compartments as measured by the chemical shift

Table 1. *pH* values of cytoplasmic and vacuolar compartments during growth of *C. albicans* in the yeast or mycelial form

Phase and form of growth*	pH value in:	
	Cytoplasm	Vacuole
Y ₈	6.7	—
Y ₂₄	6.7	6.3
Y ₄₈	6.8	5.8
GT	6.7	6.1
M	6.4	5.7

* Y, Yeast form; GT, germ tube; M, hyphae. The subscript number indicates the hours of growth in the yeast form. For other details, see Methods.

of P_i signals. Vacuolar pH, although much more variable than cytoplasmic pH, was always significantly more acidic than cytoplasmic pH, which ranged slightly below neutrality (6.7–6.8) in all cases except the hyphae, where it measured about 6.4.

DISCUSSION

We have presented ^{31}P NMR spectra of the fungal pathogen *C. albicans* at different phases of both the yeast or mycelial form of growth. These spectra indicate that significant modulations occur in the signals arising from phosphate as well as from other phosphorus-containing metabolites. The ^{31}P NMR spectra also allowed precise monitoring of intracellular pH variations.

Starting from the early-stationary phase of growth, yeast-form cells as well as germ tubes and hyphae showed two clearly resolved peaks in the inorganic phosphate region. In cells from the late-stationary phase of growth, the position of these peaks correspond to P_i at pH 6.8 (II) and 5.8 (III) (Table 1). As a rule, all samples examined had no extracellular phosphates. When phosphate was added to cells in the NMR tube, signals II and III had practically unaltered chemical shifts and a new peak at a lower field appeared (data not shown). Therefore, it is reasonable to conclude that signals II and III arise from phosphate localized within two different cellular compartments, which can be assumed to be the cytoplasm and the vacuole, respectively. It is known that phosphate is present both in the cytoplasm and in the vacuole and that the vacuolar milieu is more acidic than the cytoplasmic one (Indge, 1968; Roberts *et al.*, 1980). This interpretation is also supported by the following facts: (i) PCA-extracts showed only one signal in the P_i region; (ii) only old, stationary phase cells exhibited a strong signal III as the vacuole becomes more evident and enlarges during cell ageing in the stationary phase (Matile *et al.*, 1969). However, the vacuolar P_i signal had a lower level of detectability at certain stages of growth and mycelial conversion (see Figs 1, 3). Since at low pH values, the chemical shift of P_i signals is rather insensitive to pH variations (Roberts *et al.*, 1981), the measurement of vacuolar pH is considerably less precise than that of cytoplasmic pH (see also below).

A growth-related modulation in the intensity of the polyphosphate signal (IX) has been observed. This intensity was highest (relative to the total P_i signal) in the exponentially growing cells, then regularly declined as cells progressed to the stationary phase. At the latest time examined (48 h), the polyphosphate/ P_i ratio decreased by about one order of magnitude with respect to the exponentially growing cells. Thus, contrary to what is generally supposed (Harold, 1966), polyphosphates appear to accumulate relatively early during growth and to be disposed of when the organism slows its growth due to limitation in the carbon/energy source. The shift of the polyphosphate signal to lower field as the cells age supports the above interpretation, since it is known that short polyphosphate chains do indeed resonate at a lower field than longer ones (Navon *et al.*, 1979). When the organism was cultivated with a surplus amount of phosphate, both polyphosphate and vacuolar P_i signals increased in intensity relative to that of the cytoplasmic P_i signal suggesting a metabolic interchange between polyphosphates and free phosphate within the vacuole.

Our data also suggest that polyphosphates are subjected to a high turnover rate during yeast-to-mycelium transition in *C. albicans*. In fact, the polyphosphate signal had a very low intensity in the germ tubes and was not detected at all in the hyphae. Since hyphal growth in *N*-acetylglucosamine-medium occurs in the absence of external P_i , the exhaustion of polyphosphates reflects a need for phosphate during the transition, which cannot be satisfied by utilization of the available intracytoplasmic and vacuolar phosphate.

From the chemical shift of the cytoplasmic P_i signal, the value of the intracytoplasmic pH could be determined. It was fairly constant (6.7) throughout the growth in the yeast form and also in the germ tubes, but significantly lower (6.4) in the mycelial form (Table 1). This would indicate differences in the metabolism of the two forms, as suggested also by previous studies (Chattaway *et al.*, 1973; Land *et al.*, 1975). It must be considered that the cytoplasmic pH is strongly regulated so as to undergo minimal variation even when large variations in external pH occur (Salhany *et al.*, 1975). The cytoplasmic pH measured in this study is more alkaline than the one (6.45) measured by Chattaway *et al.* (1973) by more conventional methods in sonically disrupted cells. This discrepancy is too large to be accounted for by the limitations in the accuracy of ^{31}P NMR for the absolute pH determination *in vivo* using a single standard titration curve (Roberts *et al.*, 1981). It must be emphasized that in sonically disrupted cells the measurement of pH is inevitably affected by intermixing of cytoplasmic and vacuolar contents.

The nature of signals IV and V is unknown. Both signals do not titrate with external pH change and must arise from acid-stable compounds, since they are present in PCA-extracts. From its resonance frequency and other spectral characteristics, signal IV could represent glycerophosphorylcholine, whose presence in ^{31}P NMR spectra of *Saccharomyces cerevisiae* has already been reported (Navon *et al.*, 1979). Interestingly, the intensity of this signal was highest in stationary phase, yeast-form cells, decreased in the germ tubes and practically disappeared in the hyphae (Fig. 3*a-c*). Signal V is also centred in a spectral region where compounds with phosphodiester bonds resonate. This region may include the tRNAs (Salhany *et al.*, 1975; Navon *et al.*, 1979) and this was verified by ourselves by addition of a commercial tRNA to the cellular extracts. However, in intact cells, it might also contain other phosphodiester compounds, including cell-wall phosphomannan (Costello *et al.*, 1975).

Resonance band I was always detected, although with different intensities, in cells at any phase or form of growth. Its frequency range, centered at about +3 p.p.m., is characteristic of sugar-phosphate monoesters, mononucleotides and glycolytic intermediates (Burt *et al.*, 1979). Navon *et al.* (1979) assigned this band to 3-phosphoglyceric acid in intact resting cells of *S. cerevisiae*. PCA-extracts of *C. albicans*, as reported in this study, showed that band I comprises several, distinct peaks, the nature and relative intensities of which varied significantly with both the phase and form of growth. A preliminary analysis of these compounds was performed on PCA-extracts by addition of purified compounds. Along with a number of compounds still unidentified, extracts of yeast-form cells appeared to contain AMP and fructose 6-phosphate, while in mycelial extracts, glucose 6-phosphate and fructose 6-phosphate were identified. Overall, these findings are consistent with the assumed metabolic differences between the two forms of growth of *C. albicans* (Chattaway *et al.*, 1973; Land *et al.*, 1975), but a definite conclusion on this important aspect will be possible only when all the peaks which contribute to band I are assigned.

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