Relation between Germination, Trehalose and the Status of Water in *Phycomyces blakesleeanus* Spores as Measured by Proton-NMR

By ANDRÉ VAN LAERE,1* AN FRANCOIS,2 KRISTIN OVERLOOP,2 MARC VERBEKE3 AND LIEVEN VAN GERVEN2

1Laboratory for Plant Biochemistry, KULeuven, Kardinaal Mercierlaan 92, B-3030 Heverlee, Belgium
2Centre for Nuclear Magnetism and Radiospectroscopy, Laboratorium voor Vaste Stof Fysica en Magnetisme, KULeuven, Celestijnenlaan 200 D, B-3030 Heverlee, Belgium
3Interdisciplinary Research Centre, KULeuven, Campus Kortrijk, B-8500 Kortrijk, Belgium

(Received 29 May 1986; revised 11 August 1986)

The distribution and properties of the cellular water in sporangiospores of *Phycomyces blakesleeanus* were investigated using proton-NMR. In dormant spores different classes of water were characterized by a difference in their transverse relaxation times \( T_2 \). The amount of cytoplasmic water was estimated to be as low as about 700 mg (g dry wt)\(^{-1}\) and its small \( T_2 \) (18.2 ms) indicated a very limited mobility. About 10 min after induction of germination (by a heat shock or by addition of 0.1 M-acetate), both the content and the mobility of the cytoplasmic water increased sharply. These changes coincided with a rapid breakdown of most of the cellular trehalose and with the production (and leakage from the spores) of large amounts of glycerol. The role of these biochemical changes is discussed in relation to the water status of the spores.

INTRODUCTION

Although in nature the sporangiospores of *Phycomyces blakesleeanus* are liberated as a sticky droplet from the sporangia (Bergman et al., 1969), they are likely to be subjected to desiccation before they reach a suitable habitat for germination. Indeed the spores can support complete desiccation without any loss of viability and they are normally stored in the desiccated state in the laboratory (Furch, 1971).

Up to 35% of the spore dry weight consists of the non-reducing disaccharide trehalose (Rudolph & Ochsen, 1969). Besides an obvious role as a reserve carbohydrate, trehalose probably protects the spores from desiccation damage. Trehalose is indeed found in high concentration in anhydrobiotic organisms such as fungal spores (Sussman & Lingappa, 1969), *Artemia salina* cysts (Clegg, 1965) and the nematode *Aphelenchus avenae* (Madin & Crowe, 1975) or overwintering insects (Shimada et al., 1984). The protective effect of trehalose may largely be due to a specific role in the preservation of membrane structure in the dry state (Crowe et al., 1984) or to the stabilization of enzymes (Hecker & Sussman, 1973).

Although rehydration of the spores after suspension in water occurs in a split second, the water content of spores hydrated in water-saturated air has been reported to be as low as 50% (Verbeke & Van Laere, 1986) to 60% (Furch, 1978). With such low water contents, the availability and/or mobility of water might limit enzymic activity in the spores, especially if the substrates are large molecules (Potthast, 1978). Perhaps this effect contributes to the phenomenon of dormancy in *Phycomyces* spores. These spores indeed do not germinate even when supplied with ample water and nutrients.

NMR is a very powerful and non-destructive method for the detailed study of the physical state and distribution of water in biological systems. The most useful NMR parameters are the relaxation times (longitudinal relaxation time \( T_1 \), and transverse relaxation time, \( T_2 \)). In
of the spores. The treatment did not result in immediate changes in distribution and/or content with a refractive index detector. The glycerol content of both spore extract and filtrate was measured according to Wieland (1963).

Determined by drying the spores at 120 °C, weighing and subtracting the weight of the dry filter. 0.1 vol. determined by fitting the experimental decay curves with a sum of exponentials using a home-made multiexponential fitting program. The program is based on the least-squares procedure, which means that the condition of making the sum of the squared deviations between experimental and calculated values minimal is satisfied by finding the parameter values which minimize this sum. When there is only one relaxing component present a simple exponential decay curve is expected. The results indicated that there are multiple fractions of cell water which involve distinct relaxation times, which can be separated using a multiexponential fitting program.

Activated by adding 0.5 ml 3 M-ammonium acetate (final concn 0.1 M).

At the indicated times, 4 ml of spore suspension (40 mg) was filtered with suction on Whatman GF/C filters. Both the filtrate and the filter with the spore cake were quickly transferred to liquid nitrogen until further processing. The rest of the spore suspension (110 mg) was filtered with suction on a Millipore LSWP filter. The spore paste was placed in the bottom of a glass tube (diameter 10 mm) and subjected to NMR (see below). Plasmolysed cells were obtained by adding solid potassium chloride to saturation 2 min before filtering the spores. A cell wall fraction was prepared by homogenizing 2 g spores in 25 mM-Tris/HCl, pH 7.1. The 1000 g (2 min) precipitate was washed with the extraction buffer and subjected to NMR.

The water content of the spores was followed by filtering 5 ml (50 mg air-dry spores) on Millipore LSWP filters, weighing and subtracting the weight of a similarly wetted filter to obtain the fresh weight. Dry weight was determined by drying the spores at 120 °C, weighing and subtracting the weight of the dry filter.

The spore cake (40 mg dry wt) in the liquid nitrogen was homogenized with glass beads as described previously (Van Mulders & Van Laere, 1984) in 1 ml 1 M-HClO4. Part of the 10 000 g supernatant was neutralized with 0.1 vol. 5 M-K2CO3. The resulting KClO4 precipitate was centrifuged. Part of this supernatant was subjected to HPLC on a Lichrosorb-NH2 (5 μm) column with 70% (v/v) acetonitrile as the solvent to determine the trehalose content with a refractive index detector. The glycerol content of both spore extract and filtrate was measured according to Wieland (1963).

The NMR experiments were done with a Bruker CXP 90 MHz spectrometer equipped with a Varian electromagnet. The transverse relaxation time, T2, of the protons in the spore samples was measured by the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence (Meiboom & Gill, 1958). A typical result is shown in Fig. 1. When there is only one relaxing component present a simple exponential decay curve is expected. The results indicated that there are multiple fractions of cell water which involve distinct T2 values. Each point of the decay curve is the mean of at least 20 measurements. The T2 and the relative importance of the different fractions were determined by fitting the experimental decay curves with a sum of exponentials using a home-made multiexponential fitting program. The program is based on the least-squares procedure, which means that the condition of making the sum of the squared deviations between experimental and calculated values minimal is used in determining the parameters. The amount of water in each fraction was calculated taking into account the total water content of the spores.

All individual parts of the experiments were repeated at least three times with consistent results. The data presented stem from one self-contained experiment.

RESULTS

Different categories of water

Fig. 1 shows the spin-echo decay curve, as measured with the CPMG pulse sequence method, for fully hydrated dormant and 3 h germinated spores (A and B, respectively). These curves were non-exponential and at least four exponentials were needed to describe the pattern. This revealed the presence of different fractions of non- (or slowly) exchanging water in the spores (Table 1): fractions 1, 2 and 4 were also found in plasmolysed cells and in isolated cell walls. Since the nature of these fractions is uncertain and since their contribution does not change during germination, they are not discussed further. Fraction 3 (T2 about 18 ms), which represented about 45% of the water in dormant and up to 58% in 3 h germinated spores, was not found in cell walls and decreased sharply after plasmolysis.

Changes after heat activation

Heat activation of the spores changed the germination percentage from near 0% to over 85% of the spores. The treatment did not result in immediate changes in distribution and/or...
NMR and water in germinating spores

Fig. 1. Spin-echo decay curve of dormant (A), 3 h germinated (B) or plasmolysed (C) spores and isolated cell walls (D).

Table 1. $T_2$ (ms) and percentage of total (in parentheses) of different water fractions in dormant, 3 h germinated and plasmolysed spores of *P. blakesleeanus*

<table>
<thead>
<tr>
<th>Fraction 1</th>
<th>Fraction 2</th>
<th>Fraction 3</th>
<th>Fraction 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dormant</td>
<td>0.306 ± 0.048</td>
<td>7.0 ± 0.7</td>
<td>18.2 ± 0.25</td>
</tr>
<tr>
<td>Germinated</td>
<td>0.355 ± 0.041</td>
<td>8.2 ± 0.5</td>
<td>33.9 ± 1.9</td>
</tr>
<tr>
<td>Plasmolysed</td>
<td>0.223 (20.3%)</td>
<td>4.0 (67.1%)</td>
<td>13.7 (7%)</td>
</tr>
<tr>
<td>Cell walls</td>
<td>0.238 (8.9%)</td>
<td>8.0 (88.7%)</td>
<td>0 (2.4%)</td>
</tr>
<tr>
<td>Water</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Trehalose (2.5 M)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Agar (2.5%)</td>
<td>ND</td>
<td>ND</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(95.6%)</td>
</tr>
</tbody>
</table>

ND, Not detected.

Properties of the water. However, during early germination significant changes occurred. Between 5 and 30 min after the heat treatment, the water content of the spores nearly doubled (Fig. 2). The main change occurred in the fraction with the second largest $T_2$ (fraction 3), which increased sharply (Fig. 2) and also its $T_2$ increased significantly (Fig. 3). The quantitative changes in the other fractions were only minor (Fig. 2). No changes were found during incubation of dormant spores under the same conditions.

In an attempt to relate these changes to cellular contents, the amounts of trehalose and glycerol were measured. These are the main products in which bulk changes are known to occur during early germination. Coincident with the changes in the NMR pattern, the amount of trehalose rapidly decreased in germinating spores (Fig. 4a). Large amounts of glycerol were synthesized during the same critical time period between 5 and 30 min after heat treatment. Although this glycerol initially remained inside the cells, after about 20 min it started leaking to the surrounding medium. Again, no changes were detected during incubation of dormant spores (not shown).

Changes during acetate activation

Similar and even more pronounced changes were found shortly after ammonium acetate was added to the spores. Such treatment also induced massive germination of the spores (95%). The
water content increased from 1.7 to about 3.3 g (g dry wt)$^{-1}$ between 10 and 30 min after the start of the treatment. Again, this change was mainly due to an increase in fraction 3 with the second largest $T_2$. Also, the $T_2$ of this fraction increased greatly during the same time period (not shown). The breakdown of trehalose and the synthesis of glycerol were even more pronounced during acetate activation. Again, during the same critical period between 10 and 30 min, most of the trehalose was broken down, and large amounts of glycerol were synthesized and started leaking out of the spores after a short lag period (Fig. 4b).

**DISCUSSION**

**Spore water status**

The spin-echo decay curve indicated the presence of four different fractions of slowly exchanging protons in the spores. No major qualitative or quantitative changes occurred in fractions 1, 2 and 4 during germination. Since the nature of these fractions is rather uncertain, they will not be discussed further.
NMR and water in germinating spores

Fraction 3, with the second largest \( T_2 \), is likely to represent the bulk cytoplasmic water since it was not found in cell walls and was greatly reduced by plasmolysis. It is remarkable that fully imbibed spores contained only about 700 mg cytoplasmic water (g dry wt)^{-1}. This would result in a solution of trehalose with a concentration greater than 1 M, without taking into account other cytoplasmic solutes and proteins. It was therefore not surprising that the \( T_2 \) of this fraction was still extremely small. This is an indication of water which is greatly hindered in its mobility, perhaps to such an extent as to limit metabolic activity (compare with 2.5% agar where the water is more mobile). Hence, the increase in amount and mobility (as indicated by its increasing \( T_2 \)) of this fraction might alone stimulate metabolism, especially reactions in which larger molecules are involved (Potthast, 1978) (e.g. protein synthesis).

Role of trehalose breakdown

The obvious possibility that trehalose is a reserve carbohydrate for the germinating spores is contradicted by several observations.

1. *Phycomyces* spores do not germinate when supplied with exogenous glucose, although the compound is taken up and even metabolized to more trehalose by the dormant spores (Van Laere & Carlier, 1975).

2. Even after an activating treatment, the spores are dependent on the presence of exogenous glucose (or another suitable carbon source) for germination (Van Laere et al., 1980a). Therefore the endogenous trehalose is not able to sustain early germination, possibly because its breakdown is only temporary as is the activation of trehalase (Van Assche et al., 1972; Van Mulders & Van Laere, 1984).

3. The breakdown of trehalose is disproportionate with its role as a reserve. The breakdown is so fast that respiration cannot keep pace and large amounts are fermented to the energetically useless and even wasteful glycerol which moreover, leaks from the spores.

4. The breakdown of trehalose is stimulated by its product, glucose (Van Mulders & Van Laere, 1984), just as in *Mucor rouxii* (Dewerchin & Van Laere, 1984) and yeast (Thevelein & Jones, 1983). Moreover breakdown of trehalose is much faster during acetate activation (compare Fig. 4a and b) although acetate is a good substrate for *Phycomyces* (Van Laere et al., 1980b) and consequently, one might expect a slower breakdown of reserve. It should be noted that, contrary to yeast, where acetate induces sporulation, in *Phycomyces*, germination of the spores is induced.

Therefore trehalose does not behave like a typical reserve component and apparently the spores eliminate most of it as soon as possible. Perhaps the main role of trehalose is to protect the spores from desiccation damage by replacing the hydration water of membranes and proteins (see Introduction). However, during rewetting efficient rehydration of cellular components (especially trehalose impermeable organelles such as mitochondria) might be prevented and the availability of sufficiently mobile water might be too limited to allow a full metabolic activity. For example, mitochondria in fully hydrated dormant spores still have a peculiar lobate structure which changes to a more typical mitochondrial aspect during early germination (Pambor, 1978). A way for the spores to allow enough mobile water into the cytoplasm would be to lose much of their abundant trehalose. This is effectively what happens during the first 30 min of germination where 41–68% (Fig. 4) of the cellular trehalose disappears.

Role of glycerol synthesis

A specific role for glycerol as a ‘germination substrate’ is unlikely since most glycerol leaks from the spores and dormant spores do not germinate when the culture medium is supplemented with glycerol. However, glycerol produced from trehalose might mediate an increased osmotic pressure in the spores since apparently it does not leak from the spores during early germination. The cellular glycerol concentration reaches a mean value of about 400 mM in the spores. Moreover, glycerol concentration in individual spores might reach much higher peak values which become obscured in the analysis by lack of synchrony in the population. The concomitant increase in osmotic pressure is perhaps responsible for a plastic deformation of the spore, whereafter glycerol starts to leak out. Indeed, spores are known to swell (Galle, 1964), to take up
large amounts of water (Fig. 2) and to decrease in density (Van Laere et al., 1980c) during the first 30 min of germination. A loosening of the cell wall might also be involved in this process (Verbeke & Van Laere, 1982, 1986), but it remains difficult to distinguish between cause and effect in this respect.

Alternatively, glycerol might be a means of eliminating large amounts of trehalose in a short time. Although the spores have the enzymic equipment to produce the energetically more useful ethanol and lactate (Furch, 1973), only small amounts are produced (Furch, 1972). At high concentration, these products are potentially toxic whereas glycerol is fully compatible with enzymic activity, as exemplified by its role in several osmophilic fungi and algae (Brown, 1978). Unlike glucose (and trehalose) it can leak from the spores rather quickly without causing massive membrane leakiness. Moreover the cells have the enzymes to synthesize glycerol very rapidly by using both NADH from glycolysis and NADPH from the shunt as reductants. Indeed, glycerol phosphatase is active on both dihydroxyacetone phosphate and glycerol 3-phosphate at a comparable rate (Van Schaftingen & Van Laere, 1985) and besides an active glycerol-3-phosphate dehydrogenase (NADH dependent) an NADPH dependent glycerol oxidoreductase is also present (Van Laere, 1985).

Conclusion

That these processes are not just accidental coincidences of the germination process is shown by the fact that both the breakdown of trehalose and the synthesis of glycerol are temporarily activated (up to 10-fold) by a transient rise in cyclic AMP content which provokes an activation of the rate limiting enzymes trehalase (Van Laere & Hendrix, 1984; Van Mulders & Van Laere, 1984) and glycerol phosphatase (Van Schaftingen & Van Laere, 1985). Also glycolysis is transiently stimulated in the germinating spores by an increase in the concentration of fructose 2,6-bisphosphate (Van Laere et al., 1983), which is a potent stimulator of Phycomyces phosphofructokinase (Van Laere, 1983).

There appears to be no biochemical necessity or even rationale for these bulk changes. However, NMR data indicate that water availability and mobility are very restricted in dormant spores and that during early germination (10–30 min) both the amount and the mobility of cytoplasmic water increase together with a decrease in trehalose content. During this same period protein synthesis, RNA synthesis and respiration increase dramatically (Van Laere et al., 1980a). Therefore water might be a factor contributing to the phenomenon of dormancy in Phycomyces spores.

A. V. L. is a senior research associate of the National Fund for Scientific Research (Belgium). A. F. is a research fellow of the Inter-University Institute for Nuclear Research (Belgium). K. O. is a research assistant of the National Fund for Scientific Research (Belgium).

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