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CALORIMETRIC ASPECTS OF THE HEAT ACTIVATION OF SPORES OF PHYCOMYCES BLAKESLEEANUS

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Summary

When Phycomyces spores are heated in a differential scanning calorimeter (DSC) an exothermic process situated in the activation temperature range is detected. This process does not depend on oxygen and is absent in scans of activated spores. After activation the protein denaturation profile is also changed but this effect seems to depend on oxygen. Acetate activates spores in about 10 min, however longer treatment with this chemical is needed to affect the exothermic peak. The effects of some other chemicals that influence the activation mechanism, like D_2O and $FeCl_2$, are also reported.

Introduction

Sporangiospores of Phycomyces blakesleeanus have to be activated in order to germinate. One of the activating treatments is to heat wetted spores for 3 to 5 min at 40°C to 50°C (1). In a previous report (2) we demonstrated that as a consequence af activation, the protein denaturation profile recorded with a DSC is changed. However, in the scans no signal related to the activation itself could be found. It was suggested that in the DSC-cups the spores were not sufficiently hydrated to be activated. Therefore, we now performed experiments with a different DSC allowing the use of larger capsules, enabling the addition of more water to the spores.

Methods

Phycomyces blakesleeanus (strain 1+) was grown and harvested as described by Van Assche et al. (3). The spores were brought into 150 µl pans, wetted and the pans were closed. They were scanned in a Seteram differential scanning calorimeter with sensibility 8.5 µV/mW (according to the calibration data).

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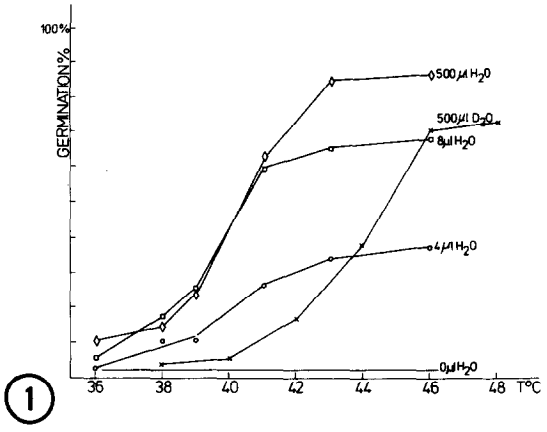


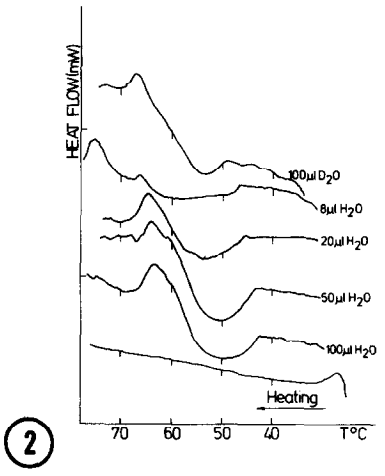


Fig. 1. Spores (6 mg) were heated for 5 min with different amounts of water or D_2O at increasing temperatures as indicated in the figure. The germination % was determined ± 12 h later in a microscope.

Fig. 2. Spores (30 mg) are scanned with different amounts of water or D_2O added to the spores as indicated in the figure. Heating rate 1 °C/min. The unmarked scan was taken with heat denaturated spores.

All scans were made at 25 µV/full scale and paper speed 3 mm/min. The distance between two scale divisions on the ordinate corresponds to 1 mW. In the Figures the curves have been displaced on the ordinate in order to show a clear picture. In germination experiments the pretreated spores were grown on culture medium for 12 h and the germination percentage determined in a microscope.

Results and Discussion

Figure 1 shows the dependency of the germination induction on degree of hydration. With 8 µl water added to 6 mg spores about the same activation curve is obtained as in excess of water. The dependency of the DSC-scans on hydration is shown in Figure 2. With 50 µl water added to 30 mg spores, which allows normal activation, indeed practically the most "pronounced" scan is obtained. At elevated temperatures the protein denaturation peak reported earlier (2) is observed. In contrast to the earlier data (2), also an exothermic process occurs, starting at 43°C-44°C. At 0.5°/min heating rate this process starts at 41°C which corresponds even better with the activation temperature. The heating rate also seems to be an important factor for the presence or abscence

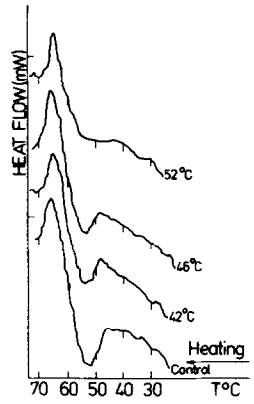
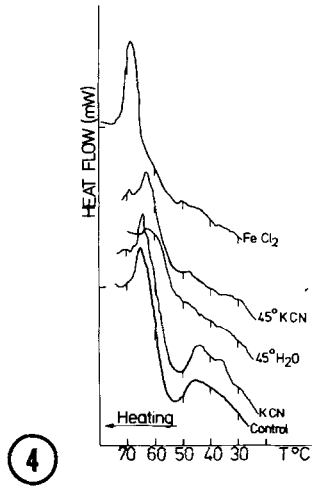


Fig. 3. Spores (30 mg) with 50 µl water were prescanned heated in the DSC up to the temperatures indicated on the curves. Then the spores were cooled and scanned again. This second scan is shown in the figure. Heating rate : 2 °C/min.

of an exothermic process in the scans. In 2°C and 3°C/min scans the occurence of the exothermic process is less pronounced, resembling in the latter case a return to the base line. The precise course of the base line however cannot be known in scans with spores. Scans of heat denaturated spores in water give a constantly increasing signal (Fig.2), due to the changing specific heat of the spore material. It is not sure however that this line is a valuable reference for normal spores. As a consequence the calculation of the heat involved in the calorimetric peaks is not possible and one cannot be absolutely sure that a decrease in heat uptake does represent the start of an exothermic process. There is however not much doubt that in our scans an exothermic process really occurs. From Figure 3 it is seen that pretreatment of the spores at increasing temperatures between 40°C and 50°C results in a stepwise disappearance of heat from the "exothermic" peak. This is logical because the process concerned is accomplished to an increasing extent during the pretreatment. Assuming e.g. that an endothermic process would take place during pretreatment (to ± 44°C) the findings from Figure 3 would be completely incomprehensible. Figures 3 and 4 also suggest that the exothermic process represents an aspect of the activation : in activated spores this process indeed no longer occurs.

We tested several chemicals that influence the activation temperature. In ger-



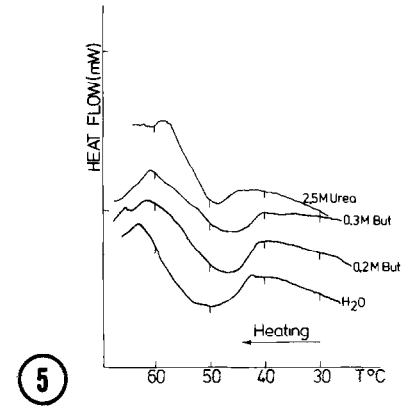


Fig. 4. Scans of 30 mg spores in 50 µl water (= control), in 50 µl of KCN 50 mg/l and in 50 µl $FeCl_2$ 1 M. The curves marked "45 °C" are scans of spores which have been pretreated 50 min at 45 °C in water or in 50 mg/l KCN as indicated. Heating rate : 2 °C/min.

Fig. 5. Spores (30 mg) are wetted with 50 µl aq.dest., 0.2 M or 0.3 M n-butanol and still another sample with 2.5 M uream solution. They were scanned at 1 °C/min.

mination experiments we found that in D_2O the sigmoid activation curve is shifted approximately 4°C (Figure 1). Now, the midpoint of the curve is situated at ± 44°C instead of 40°C-41°C as found in water. In scans with D_2O the exothermic process indeed starts 3°C-4°C higher and the protein denaturation peak is also shifted (Figure 2). As D_2O affects many other parameters (as discussed in 4) one cannot conclude that the exothermic peak reflects simply an effect on proteins. On the other hand the exothermic process seems to depend somehow on proteins, as in 2.5 M urea solution the exothermic peak is decreased (Fig. 5) and in a 4 M solution it is absent (not shown).
In D_2O the amount of heat involved in the exothermic peak is also drastically decreased. This is an indication that a hydration phenomenon may be involved in the exothermic process. n-Alcohols are known to influence the activation temperature (5). From the results in Figure 5 it can be seen that with n-butanol added to the spores the exothermic process starts at lower temperature. The amount of heat involved also decreases. This could be (partly) rela-

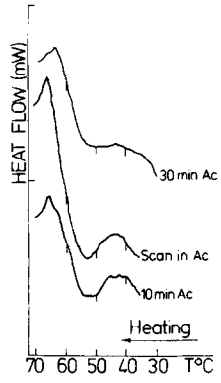


Fig. 6. The spores were pretreated for 10 min or 30 min with excess 0.1 M NH_4-acetate at 30 °C, isolated and brought into the DSC-pans. As a reference 30 mg spores were scanned directly with 50 µl NH_4-acetate solution. Heating rate : 2 °C/min.

ted to the decreased germination percentages obtained in n-butanol solutions (see 5). Thevelein et al. (5) found that in 0.3 M butanol the activation temperature is decreased by about 7°C. In DSC a much smaller effect is obtained. However in DSC pans the amount of alcohol that can interact with the spores is much smaller too. With spores pretreated in larger volumes of 0.2 M butanol at 36°C, the exothermic peak was absent as expected for activated spores (not shown). Similarly the addition of 50 µl 0.1 M NH_4-acetate to the spores in the pans does not affect the scans, while pretreatment in excess solution is effective (Figure 6). The pretreatment during 10 min suffices in order to obtain maximum activation (6) and results in a pronounced decrease of the protein denaturation peak as reported earlier (2). Longer incubations in acetate however are needed in order to obtain the disappearance of the exothermic peak (see Figure 6). Possibly the metabolism has to intervene in order to obtain that condition which is reached immediately by heat shock.

In Figure 6 (as in Figure 3) the problem arises why in scans, where the spores are also brought at activating temperatures, there is no clear effect on the protein denaturation peak as observed after pretreatments in waterbath. In the experiments from Figure 3 it can be seen that heating (and cooling) spores so that they remain 12 min between 40°C and 50°C, does not cause the drastic ef-

fect that is obtained by heating 5 min at 45°C in waterbath (see Figure 4). A major difference between the two types of experiments is the availability of oxygen. In DSC-pans the packed spores remain in nearly anaerobic conditions. Oxygen therefore could play a role in the conformational change of the proteins. This is not in contradiction with the known independency of heat activation towards oxygen. Indeed, after heat shock in absence of oxygen, the protein conformational change probably occurs at the moment when oxygen becomes available (during culture). In accordance with a role of oxygen, it is found that in scans with $FeCl_2$ solutions the protein peak is still higher than in water (Figure 4). Here, during the heating, complete anaerobic conditions exist due to the oxidation of Fe^{2+}. The absence of an exothermic peak in that scan points to the activating effect of Fe^{2+}-solutions. Treatment of spores in e.g. 1 M $FeCl_2$ for 5 min indeed results in about 35 % activation. After longer incubation in such concentrated solutions less spores germinate, but this is due to denaturating effects of the acid solution. We also studied the effects of prolonged treatments with less concentrated Fe^{2+}-solutions. After 1 h incubation an "equilibrium" seems to be reached : 0.001 M, 0.01 M and 0.1 M give resp. ± 2 %, ± 8 % and ± 13 % activation. The effect of Fe^{2+} is probably obtained through a redox reaction. Reducing agents are known to activate spores (7). If our results represent an equilibrium between Fe^{2+}/Fe^{3+} and activated/dormant spores the redox potential of the activation sites could be estimated according to the Nernst equation (the solubility product of $Fe(OH)_3$ being known). At present this knowledge however seems not very relevant. On the other hand the results with Fe^{2+} suggest that the activation site may be situated at the outside of the spores (cell wall or plasmalemma). It seems indeed improbable that Fe^{2+} would easily penetrate the cell membrane. The spores were also seen to form clusters in Fe^{2+} solutions, again suggesting an interaction of Fe^{2+} with the wall. Much further work is needed in order to confirm this assumption and to understand the activation mechanism. The exothermic process in fact is not understood. It does not simply reflect respira-

tion. Besides, scans were not affected by KCN, antimycin A, monoiodocetic acid, etc. On the other hand when spores were pretreated at 45 °C in waterbath in the presence of KCN, the effect on the protein denaturation peak was less pronounced than in the absence of KCN (see Figure 4). Therefore the protein conformational change seems to depend not only on oxygen, but also on respiration. The exothermic peak on the other hand seems not to be related with respiration at all. However, which process then is involved is unclear.

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