Isopropyl-substituted Phenols Have a Different Effect from Other Phenols on the Breaking of Dormancy by Heat Shock in *Phycomyces blakesleeanus* Spores

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Phenols are able to lower the temperature of heat activation (breaking of dormancy by heat shock) in *Phycomyces blakesleeanus* spores. The effect was observed with a series of phenols ranging, according to increasing lipophilic character, from unsubstituted phenol to 2,4-dichlorophenol. However, the concentration required to produce the same effect with each phenol was reduced with increasing apolar character. A linear relationship was obtained between the log of the concentration of each phenol needed to produce a 4°C shift of the half-activation temperature and the log of its octanol/water partition coefficient. In contrast, the isopropyl-substituted phenols thymol, 2- and 4-isopropylphenol and 3-isopropylcatechol all raised the half-activation temperature of the spores. The same effect was observed with menthol, the unsaturated analogue of thymol. The heat resistance of the spores was lowered by all phenols, including isopropyl-substituted phenols. Although the reason for the anomalous behaviour of isopropyl-substituted phenols is not known, the opposite effect on spore heat activation and spore heat resistance indicates that the activation process of the spores is not linked to the process of spore killing. Therefore, spore activation is not due to some kind of non-specific sublethal protein denaturation, as might have been concluded previously from the fact that many spore activation methods are sublethal treatments.

INTRODUCTION

Dormancy in fungal spores can be broken by a variety of treatments. One of the most intensively studied activation treatments is heat activation (Sussman, 1976). The mechanism of heat activation is not yet fully understood. Recently, however, we have shown that the first process involved is a protein conformational change and not a phospholipid phase transition (Thevelein et al., 1979).

Germination-inducing effects of organic solvents on fungal spores have been described previously (Sussman, 1965, 1976). However, a detailed study of the effect of organic solvents on fungal spore heat activation has only been made for a series of n-alcohols (Thevelein et al., 1979). In the present paper, this has been extended to a series of phenols and isopropyl-substituted phenols.

METHODS

Strain K 1+ (formerly 1+) of *Phycomyces blakesleeanus* Burgeff was used for all experiments. It was cultured on sterile moistened brown bread in the dark at 18°C and 70% relative humidity. Spores were harvested, dried and stored as described previously (Van Assche et al., 1972). Spores were used within 2 weeks of preparation. After
activation for 3 min at 50 °C in the absence of phenols, more than 90% of the spores germinated. Activation was performed with 3 mg dried spores suspended in 1 ml 0.1 M potassium phosphate buffer, pH 6.5, in the presence or absence of phenols. The suspensions were continuously shaken in test tubes in a water bath at the indicated temperatures. After the heat treatment, the spores were quickly chilled in an ice bath, collected by centrifugation and washed twice with distilled water.

After washing, the spores were immediately transferred to minimal nutrient medium. After about 8 h incubation in minimal nutrient medium, the percentage germination was determined under a microscope: 200-300 spores were counted each time. Incubation was carried out in small flasks in a reciprocating shaker at 22 °C at a spore concentration of 2 mg ml⁻¹. The composition of the minimal nutrient medium was as described by Rudolph (1958).

For a given treatment, the temperature at which 50% of the spore population germinated after heating for 3 min at sub-optimum temperatures was defined as the half-activation temperature. The temperature at which only 50% germination occurred after heating at supra-optimum temperatures was defined as the half-denaturation temperature.

All experiments were repeated at least twice. When the experiments were repeated with different spore crops, quantitative differences were observed but qualitatively the results were always consistent. Series of germination percentages, as represented by the activation or denaturation curves, were compared rather than single experimental values of percentage germination.

RESULTS

When spores were heated at different temperatures in the presence of phenols, the sigmoidal activation curve which is normally obtained between 40 and 50 °C shifted toward lower temperatures. This effect was seen with a series of phenols ranging, according to increasing lipophilic character, from phenol to 2,4-dichlorophenol. Typical examples are shown in Fig. 1 with unsubstituted phenol and 4-methylphenol. In the presence of phenols both the half-activation temperature and the half-denaturation temperature were lowered. The maximum germination percentage, obtained at the optimal heat treatment temperature, became lower with increasing phenol concentration.

Although all phenols (except isopropyl-substituted derivatives) lowered the half-activation temperature, the concentration required to produce the same effect decreased with increasing aliphalic character. A linear relationship was obtained between the log of the concentration of each phenol needed to produce a 4 °C shift in the half-activation temperature and the log of its

Fig. 1. Percentage germination as a function of the temperature of heat treatment in the presence of different concentrations of (a) phenol (●, control; △, 50 mM; ▲, 100 mM) and (b) 4-methylphenol (●, control; △, 23 mM; ▲, 35 mM).
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Fig. 2. Plot of the log of the concentration of each phenol derivative needed to produce a 4 °C shift in activation temperature vs. the log of the octanol/water partition coefficient ($\alpha$) for each derivative. The derivatives are denoted as follows: 4-F, 4-fluorophenol; 4-CH$_3$, 4-methylphenol; 4-NO$_2$, 4-nitrophenol; 3,4-diCH$_3$, 3,4-dimethylphenol; 4-Cl, 4-chlorophenol; 4-Br, 4-bromophenol; 2,4-diCl, 2,4-dichlorophenol.

Fig. 3. Percentage germination as a function of the temperature of heat treatment in the presence of different concentrations of (a) menthol (●, control; ▲, 2 mM; △, 3 mM) and (b) 4-isopropylphenol (●, control; ▲, 2.5 mM).

octanol/water partition coefficient (Fig. 2). The slope of this line was about the same as the slope of the line previously obtained for a series of n-alcohols (Thevelein et al., 1979).

In contrast to all other phenols investigated, isopropyl-substituted phenols raised the half-activation temperature. Spore resistance, however, was lowered, as was observed with the other phenols. Results like these were obtained with thymol, 2- and 4-isopropylphenol, 3-isopropylcatechol and menthol, the unsaturated analogue of thymol. Typical examples are shown in Fig. 3(a) (menthol) and Fig. 3(b) (4-isopropylphenol). In Fig. 4 the effect of 2.5 mM catechol is compared to that of 2.5 mM-3-isopropylcatechol: addition of the isopropyl group...
clearly changed the effect on the half-activation temperature. The opposite effect of isopropyl-substituted phenols on spore activation and spore denaturation resulted in progressive lowering of maximum spore germination with increasing concentration of the phenol. A similar effect was observed previously for the heat activation and heat denaturation temperature of trehalase in *P. blakesleeanus* spores in the presence of long-chain alcohols and more apolar phenols (Thevelein *et al.*, 1981).
The increase of the half-activation temperature by isopropyl-substituted phenols was truly due to suppression of activation and not to greater denaturation at lower temperatures. This was concluded from control experiments in which the normal germination medium was supplemented with 50 mM-ammonium acetate in order to determine viability of the spores. In the presence of acetate, the complete spore population is activated and therefore in a medium supplemented with acetate all viable spores germinate (Borchert, 1962). In Fig. 5 the results are shown of experiments in which the spores were activated in the presence of thymol and thereafter transferred to minimal nutrient medium with or without ammonium acetate. It can be seen that at temperatures where thymol suppressed spore activation (for 2 mM, 40–50 °C; for 3 mM, 44–47 °C), spore viability was not changed significantly. The experiment with 3 mM-thymol clearly indicated that thymol stimulated spore denaturation (Fig. 5b). It can also be seen in Fig. 5 that after activation at supra-optimum temperatures in control experiments, part of the spore population which did not germinate in minimal nutrient medium was still able to germinate in the same medium supplemented with acetate.

**DISCUSSION**

This study extends our previous observation (Thevelein et al., 1979) that n-alcohols lower the heat activation temperature range of *P. blakesleeanus* spores. A series of phenols gave a linear relationship similar to that for the n-alcohols between their effectiveness in lowering the half-activation temperature and their lipophilicity. Such a correlation between the log of the concentration of a compound necessary to cause an effect and the log of its octanol/water partition coefficient is usually taken as an indication of a hydrophobic action site (Schneider, 1968; Hansch & Dunn, 1972; Seeman, 1972). However, as pointed out previously (Thevelein et al., 1979), the slope of the line obtained for the relation between effectiveness and lipophilicity for both phenols and alcohols lies between values typical for proteins as the action site and values typical for phospholipids. Therefore, based on these data it is not possible to make a distinction between proteins and phospholipids as the primary action site of spore heat activation. However, from the effect of high pressure on spore heat activation we have been able to conclude that the primary target site of spore heat activation is a protein, and that the protein involved undergoes a conformational change (Thevelein et al., 1979). The effect of phenols reported here is a further indication of the hydrophobic nature of the protein involved in the activation process. This protein might be a membrane protein.

Dormancy in fungal spores can be broken by a variety of treatments, many of which are sublethal. For heat activation, the temperature at which maximal spore activation is obtained is only slightly lower than the temperature at which spore killing starts to occur (Goddard, 1935; Sommer & Halbsguth, 1957; Cotter & Raper, 1966; Mills & Eilers, 1973). When the activation/denaturation curve is shifted towards lower temperatures by, for example, alcohols and high pressure (Thevelein et al., 1979) or by phenols, as shown in this paper, the maximum percentage germination usually becomes lower. Thus spore activation and spore denaturation may overlap each other, so that the heat treatment resulting in a maximum percentage of spore germination represents the best compromise between activation and denaturation. Similarly, when the spore activation temperature and the spore denaturation temperature are shifted towards each other under the influence of isopropyl-substituted phenols, increasing overlap occurs between the activation and denaturation curves when the phenol concentration is raised. This results in a progressive reduction of the maximum percentage of spore germination obtained at the optimal heat treatment temperature. The effect of isopropyl-substituted phenols can also be described as a simple reduction of the extent of activation at all temperatures, but the question still remains why there is less activation at a given temperature. Our interpretation, that the overall suppression of the extent of activation is caused by an upward shift of the activation curve and a downward shift of the denaturation curve, is corroborated by the data of Fig. 5(a, b). These results clearly demonstrate that the viability of the spores in the presence of isopropyl-substituted phenols is unaltered at the lower temperatures giving activation and is lower at the higher temperatures giving denaturation.
Other sublethal activation methods include: activation by γ-irradiation (Hashimoto & Yanagisawa, 1970; Khoury et al., 1970; Hashimoto, 1971; Van Assche et al., 1977), dithionite and other reducing substances (Van Assche et al., 1978), organic solvents (Sussman, 1953; Dobbs & Hinson, 1953; Emerson, 1954; Sussman et al., 1959), urea and other protein-denaturing compounds (Cotter & O'Connell, 1976; Cotter et al., 1976; Cotter, 1979). With all these activation methods, activation conditions which are slightly supraoptimal result in decreased spore viability. In several instances optimal activation conditions are already the best compromise between spore activation and spore killing.

The fact that many spore activation methods are sublethal treatments could indicate that spore activation is related to spore killing: it has been suggested previously that the action site of spore heat activation is also the site most easily damaged by heat. Based on this assumption, the observation that the mitochondrial membrane was the first structure visibly damaged after supraoptimal heating was taken as evidence for mitochondrial involvement in spore activation (Cotter & George, 1975). However, our results showing opposing effects of isopropyl-substituted phenols on the range of heat activation and heat denaturation temperatures suggest that two different processes are involved.

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REFERENCES

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