Pyruvate Metabolism by Mitochondria from Dormant and Activated
Phycomyces blakesleeanus Spores

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Activation of Phycomyces blakesleeanus spores resulted in the production of large amounts
of pyruvate and 2-oxoglutarate. Incubation of dormant spores in 0.1 M-pyruvate at pH 3
resulted in a high internal pyruvate concentration but no 2-oxoglutarate was formed and
neither germination nor respiration was stimulated. The capacity of isolated mitochondria to
decarboxylate [1-14C]pyruvate was doubled by heat activation of the spores. This difference
in activity disappeared when the mitochondria were subjected to treatments attacking the
integrity of the mitochondrial membrane (detergent, resuspension in buffer without osmotic
stabilizer). The increase in pyruvate decarboxylating activity was found only after heating the
spores at temperatures also triggering germination of the spores. Pyruvate uptake by the
mitochondria seems, therefore, to be a limiting factor in dormant spore metabolism.

INTRODUCTION

Without activation, germination of Phycomyces sporangiospores in a complete culture
medium is limited to some 3% of the population (Sommer & Halbsguth, 1957). The
constitutively dormant spores can be activated by several treatments, heating for some
minutes at 45 to 50 °C or treatment with acetate (Robbins et al., 1942) being most reliable.
One of the prominent features of activated spores is a nearly tenfold increase in trehalase
activity (Van Assche et al., 1972; Delvaux, 1973) and a concomitant breakdown of
endogenous trehalose (Rudolph & Ochsen, 1969).

Another feature of activated spores is a rapid increase in respiration. Simultaneously
several glycolytic products (pyruvate, lactate, glycerol) are produced and excreted by the
spores (Rudolph et al., 1966; Furch, 1972; Delvaux, 1973; Furch et al., 1976). However, the
increased trehalose and glucose breakdown are not believed to be triggers of spore activation.
Indeed, addition of pyruvate to the spores under conditions where it is taken up (at pH 3)
does not stimulate germination or respiration of the spores (Van Laere et al., 1980). In
contrast, addition of acetate results in rapid respiration (faster than after heat activation) and
germination of the spores (Borchert, 1962; Van Laere et al., 1980). Since this suggests that
pyruvate breakdown might be a limiting factor, the metabolism of pyruvate by intact spores
and by mitochondrial fractions before and after activation was investigated.

METHODS

All experiments were done with the 1' strain of Phycomyces blakesleeanus Burgeff from the Halbsguth
collection. Spores were grown and harvested as described by Van Assche et al. (1972). All experiments were done

Abbreviations: DTT, Dithiothreitol; TPP, thiamin pyrophosphate.
on spores preincubated for 1 h in standard culture medium (Rudolph, 1958) at 25 °C. Activation of the spores was done by shaking a spore suspension in a water bath at 50 °C (except when stated otherwise).

For the determination of oxo acids, 50 mg spores were vibrated for 4 min at an amplitude of 7 mm in a Teflon container together with 3-5 g glass beads of diameter 1 mm and 1-5 g glass beads of diameter 3 mm using a Braun microdisembrator, in 1-5 ml of a mixture containing 3 vol. 1 M-HClO₄ and 1 vol. 0-1% (w/v) 2,4-dinitrophenylhydrazine in 2 M-HCl. After incubation for 30 min at 25 °C the homogenate was centrifuged for 2 min at 10000 g. A 1 ml portion of the supernatant was extracted twice with 2 ml ethyl acetate. The pooled ethyl acetate fractions were re-extracted twice with 2 ml 5% (w/v) Na₂CO₃. After acidification of the Na₂CO₃ fraction with 0-45 ml conc. HCl, the acidic 2,4-dinitrophenylhydrazones were extracted again with 2 ml ethyl acetate. A 1 ml portion was evaporated to dryness with a stream of nitrogen and subjected to HPLC (as below). A 1 ml portion of culture medium was treated with 0-33 ml 0.1% (w/v) Na₂CO₃. After acidification of the Na₂CO₃ fraction with 0-45 ml conc. HCl, the acidic 2,4-dinitrophenylhydrazones were extracted again as described for the spores. They were further separated using HPLC (Waters Ass.). A Lichrosorb-NH₂ (5 μm) column (300 × 4·1 mm) was eluted with 50% (v/v) aqueous acetonitrile containing 2% (v/v) formic acid. Peaks were detected at 375 nm (on a Pye Unicam instrument) and quantified by means of a Hewlett Packard 3385A integrator and external standard calibration.

Mitochondria were isolated by vibrating 100 mg spores for 2 min at an amplitude of 4 mm (amount of glass beads as described above) in 1-5 ml 50 mM-sodium phosphate buffer pH 7-38 containing 1 M-sucrose. After centrifugation for 1 min at 15000 g the supernatant was layered on 40% (w/v) sucrose in the same buffer and centrifuged for 15 min at 85000 g in a swing-out rotor. If the mitochondria could be isolated in the same buffer (experiments with Triton X-100), 0-5 ml 70% sucrose in the same buffer was layered on the bottom of the tube to prevent pelleting of the mitochondria. The mitochondrial fraction was taken from the surface of the 70% sucrose layer or resuspended from the bottom of the tube in buffer with or without 1 M-sucrose.

To evaluate the density gradient distribution of the pyruvate decarboxylating activity, spores were homogenized in 1-5 ml sodium phosphate buffer pH 7-38 for 4 min at an amplitude of 7 mm with the same amount of glass beads. The absence of osmotic stabilizer and the more vigorous homogenizing conditions were expected to disrupt the mitochondria. Indeed, after centrifugation for 4 min at 10000 g (Eppendorf microcentrifuge) essentially all pyruvate decarboxylating activity was recovered from the almost clear supernatant. A fraction of this supernatant was layered on linear 12 to 30% sucrose gradients in the same buffer and centrifuged for 3 h at 150000 g in a swing-out rotor at 1 °C. Fractions were collected with an Isco gradient fractionator and assayed for pyruvate decarboxylation as described, except that MgSO₄ was added to a final concentration of 10 mM.

The assay of pyruvate decarboxylation was done in stoppered 25 ml Erlenmeyer flasks with a centre well containing 0-5 ml 1 M-NaOH, and in the main compartment 0-75 ml mitochondrial suspension, 0-02 ml 10 mM-TPP, 0-05 ml 2 mM-CoA in 10 mM-DTT, 0-1 ml 20 mM-NAD⁺, 0-005 ml 0-1 M-pyruvate, 0-005 ml (50 nCi) [1-14C]pyruvate (sp. act. 17-3 mCi mmol⁻¹; 640 MBq mmol⁻¹) and water to make 1 ml. After 30 min incubation at 25 °C, 1 ml 0-5 M-H₂SO₄ was added to the main compartment, and after 30 min shaking, the NaOH solution was taken from the centre well and counted for radioactivity.

Fixation of mitochondria for electron microscopy was done in extraction buffer containing 2-5% (w/v) glutaraldehyde followed by 2% (w/v) OsO₄ in the same buffer. Dehydration was done with a graded acetone series; the material was then embedded in Araldite, thin sectioned, stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed in a Zeiss EM9 electron microscope.

All experiments were repeated at least three times with consistent results. The results in Tables 2 and 3 and Fig. 4 are supplemented with the standard deviation of the mean.

RESULTS
Oxo acid determination

Since pyruvate metabolism could be an important factor in spore dormancy and since the published data on pyruvate production (Rudolph et al., 1966; Furch, 1972; Delvaux, 1973) are not completely unequivocal, the amount of pyruvate and 2-oxoglutarate in spores and culture medium was determined (Fig. 1). The pyruvate concentration of heat-activated spores increased more than tenfold during the first 30 min of germination, and then decreased slightly. Concomitantly large amounts of pyruvate leaked into the surrounding medium, reaching a nearly maximal value after 30 min. Comparable amounts of 2-oxoglutarate were found in the spores, with a maximum at 10 min; 2-oxoglutarate also leaked into the medium, yielding near-maximum amounts after 20 min germination. Acetate activation caused a somewhat slower production of both pyruvate and 2-oxoglutarate (Fig. 1). However, in this case the amount of pyruvate in spores and medium was much higher than after heat
Phycomyces mitochondria pyruvate metabolism

Fig. 1. Pyruvate and 2-oxoglutarate in medium (a) and spores (b): closed symbols for heat-activated spores (O, pyruvate; ▲, 2-oxoglutarate); open symbols for spores in the presence of 0.1 M-ammonium acetate (○, pyruvate; △, 2-oxoglutarate). Point D represents dormant spores preincubated for 1 h; point A represents spores immediately after heat activation.

activation; this could be due to competition by acetate for the available enzyme systems, as it is a good substrate for spores. Some glyoxylic acid leaked into the medium during acetate treatment [about 2 nmol (mg spores)\(^{-1}\) after 1 h] but not after heat activation. Only trace amounts of oxaloacetate were detected in the spores throughout the experimental period. Breakdown of oxaloacetate to pyruvate can probably be excluded since the spores were homogenized in a medium containing 2,4-dinitrophenylhydrazine, which rapidly transforms the oxo acids to their more stable dinitrophenylhydrazones. Similar treatment of commercial oxaloacetic acid yields negligible amounts of pyruvate which could have been present in the initial product.

Since the analytical method yielded reproducible results, it was further used to check how efficiently external pyruvate could be taken up by the spores. Incubation of the spores in culture medium with 0.1 M-pyruvate did not yield an increase in pyruvate content of the spores. However, by lowering the pH to 3 (with \(\text{H}_2\text{PO}_4\)) a preincubation of 1 h with 0.1 M-pyruvate yielded a pyruvate concentration of 2.8 nmol (mg spores\(^{-1}\) which was roughly maintained—2.5 nmol (mg spores\(^{-1}\)—after 1 h incubation in culture medium. Since under these circumstances no increase in 2-oxoglutarate concentration could be detected and neither respiration nor germination was stimulated, pyruvate metabolism rather than uptake seemed to be a limiting factor in the dormant spores.

Isolation of intact mitochondria

Since pyruvate breakdown occurs in the mitochondria, we attempted to isolate these organelles from the spores. The isolation of intact mitochondria from fungal spores is complicated by the rather harsh conditions needed to break the hard spore walls. Routine homogenization of the spores was done by vibrating the spores for 4 min at an amplitude of 7 mm in a Teflon container together with glass beads. Under these circumstances most of the spores were broken but hardly any pyruvate dehydrogenase activity was found in the mitochondrial pellet, probably due to the damage done to mitochondria during homogenization. Homogenization was optimized by reducing the duration to 2 min and the
amplitude to 4 mm. After such treatment only 48 to 58% of the spores were broken as judged microscopically. From the extracted pyruvate dehydrogenase activity, 41 to 53% was recovered in the supernatant and 45 to 54% was found in the 'mitochondrial' pellet (also positive for succinate dehydrogenase).

Various sucrose concentrations in the homogenization buffer were tested. The 'mitochondrial' fraction contained considerable amounts (38\%) of the extracted pyruvate decarboxylating activity only with sucrose at 0.7 M or above. Extraction with buffers containing 1 M-sucrose yielded even better results (45 to 54\% of the extracted activity in the 'mitochondrial' fraction). Although the spores contain up to 35\% trehalose on a dry weight basis (Rudolph & Ochsen, 1969), the effect of the increased sucrose concentration might not result only from the increased osmolarity of the medium (reducing bursting of the mitochondria): the increased viscosity of the medium could have reduced the shearing forces and consequent damage to the mitochondria during the rather drastic homogenization procedure.

The presence of intact mitochondria in our preparations was suggested by the presence of about 50\% of the activity of pyruvate dehydrogenase, a matrix enzyme (Ošík, 1968; Palmer & Hall, 1972; Addink et al., 1972), in the mitochondrial pellet. It was supported by the observation that the pyruvate decarboxylation in these preparations was stimulated by Triton X-100 (see Table 2) or by bursting the mitochondria (see Table 3). Indeed, this suggested that membranes were limiting pyruvate metabolism and at least some mitochondria were intact. Although the preparation was far from pure, the presence of intact mitochondria was confirmed by electron microscopy (Fig. 2). The crenated appearance of the mitochondria probably reflected the situation in vivo, as described by Pambor (1979) in thin sections and freeze-etch replicas. The peculiar mitochondrial structure seems to be characteristic for Phycomyces spores since it is not found in mycelium or other fungal spores.

Decarboxylation of pyruvate by isolated mitochondria

$^{14}$CO$_2$ production from [1-$^{14}$C]pyruvate is the most sensitive method for measuring pyruvate metabolism by isolated mitochondria. Moreover, it is well suited for measuring pyruvate dehydrogenase activity, provided no pyruvate decarboxylase is present. Phycomyces spores probably contain little pyruvate decarboxylase activity, since negligible
Phycomyces mitochondriaal pyruvate metabolism

Fig. 3. Centrifugation of a P. blakesleeanus spore extract on a linear 12 to 30% (w/v) sucrose gradient. Continuous line, $A_{254}$; ○, pyruvate decarboxylating activity. The arrow indicates the position of the large ribosomal subunit.

production of acetaldehyde (Rudolph, 1960) or ethanol (Furch, 1972) occurs in the spores after a normal activating treatment (3 min at 50 °C) although considerable amounts of pyruvate (Rudolph et al., 1966), lactate (Furch, 1972) and glycerol (Furch et al., 1976) accumulated under those conditions. In contrast, when treated at higher temperatures (54 or 56 °C) the spores produce acetaldehyde (Rudolph, 1960) and ethanol (Furch, 1972). These high temperatures, however, start destroying the spores and could well affect the integrity of the mitochondria and the pyruvate dehydrogenase complex. Furch (1973) described pyruvate decarboxylase in Phycomyces spore extracts. However, it is not clear from the description of the method whether a distinction could be made between pyruvate decarboxylase and the abundantly present lactate dehydrogenase.

In order to ascertain that the $^{14}$CO$_2$ production from [1-$^{14}$C]pyruvate was caused by the activity of pyruvate dehydrogenase and not by pyruvate decarboxylase, a crude extract was prepared from spores. Preparation of the extract in the absence of osmotic stabilizer and more vigorous homogenizing conditions resulted in the appearance of essentially all pyruvate decarboxylating activity in a 10000 g supernatant. After density-gradient centrifugation of this supernatant the $A_{254}$ profile showed a first peak containing soluble materials, a second peak corresponding to the small ribosomal subunit, and a third peak corresponding to the large ribosomal subunit (shoulder) and the ribosomes (which started pelleting under these conditions) (Fig. 3). The identity of the peaks was confirmed in independent experiments by the coincidence of incorporated labelled uridine with the putative ribosomal peaks. Nearly 40% of the pyruvate decarboxylating activity layered on the gradient was recovered in the different fractions, with a predominant peak in the neighbourhood of the large ribosomal subunit (about 60S) (Fig. 3). No significant activity was recovered from the pellet. The activity therefore sedimented as expected for a large multi-enzyme system like the pyruvate dehydrogenase complex of other organisms. The S value for pyruvate dehydrogenase varies, from 47-5S in pea (Reid et al., 1977) to over 59S in broccoli (Rubin & Randall, 1977) and 64S in Escherichia coli (Reed & Willms, 1966), and up to 85S in Neurospora crassa (Harding et al., 1970) and similar values in other sources (Reed, 1974).

$^{14}$CO$_2$ production from [1-$^{14}$C]pyruvate in extracts was largely inhibited in the absence of added CoA and TPP, as expected for pyruvate dehydrogenase (Table 1). Omission of NAD$^+$ had only a small effect, probably due to the rapid reoxidation of NADH in the extracts. Mg$^{2+}$ (10 mM) had no effect (101.4% ± 9.5 [S.D.]) on the activity of intact mitochondria and
Table 1. Decarboxylation of pyruvate by an extract (without sucrose) of dormant spores

Complete assay mixture (see Methods) gave an activity of 4.1 nmol min$^{-1}$ (mg protein)$^{-1}$. The results are expressed as a percentage of this value.

<table>
<thead>
<tr>
<th>Assay mixture</th>
<th>Activity (%)</th>
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<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>Minus TPP</td>
<td>13.5</td>
</tr>
<tr>
<td>Minus NAD$^+$</td>
<td>65.4</td>
</tr>
<tr>
<td>Minus CoA</td>
<td>21.2</td>
</tr>
<tr>
<td>Plus 20% sucrose</td>
<td>102.5</td>
</tr>
<tr>
<td>Plus 40% sucrose</td>
<td>80.9</td>
</tr>
<tr>
<td>Plus 0.2% Triton X-100</td>
<td>86.8</td>
</tr>
</tbody>
</table>

Table 2. $^{14}$CO$_2$ production from [1-$^{14}$C]pyruvate by mitochondria from dormant and activated spores and the effect of Triton X-100

<table>
<thead>
<tr>
<th>Spores</th>
<th>Activity [nmol min$^{-1}$ (mg protein)$^{-1}$]</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Dormant</td>
<td>1.79 ± 0.13 (100%)</td>
</tr>
<tr>
<td>Activated</td>
<td>3.39 ± 0.18 (100%)</td>
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</tbody>
</table>

yielded only limited stimulation (116.6% ± 7.2) in broken mitochondria, suggesting that sufficient Mg$^{2+}$ was present in the mitochondrial fractions. $^{14}$CO$_2$ production was nearly linear over the 30 min assay period and good proportionality was found between $^{14}$CO$_2$ production and enzyme concentration. Blanks showed negligible activity.

Mitochondria from dormant and activated spores

Since we suspected that pyruvate uptake by the mitochondria could be a limiting factor, pyruvate decarboxylation by the mitochondria from dormant and activated spores was assayed in the absence and presence of Triton X-100 (Table 2). Addition of Triton X-100 (which slightly inhibited pyruvate dehydrogenase activity) almost doubled pyruvate decarboxylation by mitochondria from dormant spores. Mitochondria isolated from heat-activated spores had a much higher control activity and were not significantly stimulated by the addition of Triton X-100. This suggested that pyruvate uptake was a limiting factor in pyruvate metabolism by mitochondria in the dormant spores, but not in activated spores.

Similar results were obtained after destruction of mitochondrial integrity by resuspension in buffer without osmotic stabilizer (Table 3). Again, bursting of dormant mitochondria increased the activity of the preparation, while the same treatment on activated mitochondria had no significant effect. The control activity of mitochondria from activated spores was also much larger than that of dormant spores and comparable to the activity found after lysis of dormant mitochondria.

To ascertain that the effects described were not just heat mediated and not related to the activation of the spores, the experiments were repeated at different activating temperatures and the subsequent germination of the spores was determined. As shown in Fig. 4, the pyruvate decarboxylating activity of the mitochondria started to increase together with the increase in germination from 42°C. The activity after treatment with Triton X-100 was not significantly affected by treating the spores at different temperatures.

Similar results were obtained when mitochondria were lysed by resuspension in buffer without osmotic stabilizer (not shown). At 48°C germination was maximal, but heating at
Table 3. $^{14}$CO$_2$ production from [1-$^{14}$C]pyruvate by mitochondria from dormant and activated spores

<table>
<thead>
<tr>
<th>Spores</th>
<th>Activity [nmol min$^{-1}$ (mg protein)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resuspended in buffer with 1 M-sucrose</td>
</tr>
<tr>
<td>Dormant</td>
<td>1.54 ± 0.26 (100%)</td>
</tr>
<tr>
<td>Activated</td>
<td>2.48 ± 0.26 (100%)</td>
</tr>
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</table>

Fig. 4. Pyruvate decarboxylation by a mitochondrial fraction prepared from P. blakesleeanus spores after heating the spores for 3 min at different temperatures. ▼, Control activity; ▲, activity in the presence of 0.2% Triton X-100; ■, percentage germination of the spores after a corresponding heat treatment. The bars represent standard deviations.

50 °C resulted in a further increase in the pyruvate decarboxylating activity of the intact mitochondria. Since the activity after lysis tended to decline at 50 °C, this might reflect incipient damage to the mitochondria. Indeed, higher temperatures reduced spore germination and viability.

**DISCUSSION**

The activation of Phycomyces spores in the presence of acetate and the inhibition of this activation by azide (Van Laere et al., 1980) suggested that, at least for activation by acetate, mitochondrial metabolism was a critical factor. Since, on the other hand, respiration quickly and dramatically increased after addition of acetate to dormant spores, the further metabolism of acetate by the spores did not seem to be a limiting factor. Also, Keyhani et al. (1972) showed that in dormant Phycomyces spores the complete cytochrome system was present.

Addition of pyruvate, however, did not stimulate spore germination or respiration (Van Laere et al., 1980) although our results show that, at least at pH 3, pyruvate penetrates into the spores. However, no 2-oxoglutarate synthesis was observed after loading the spores with pyruvate. Since acetate is quickly metabolized by the spores, pyruvate metabolism is probably rate limiting in dormant spore metabolism. The two possible limiting steps between
acetate metabolism, which is fast, and pyruvate metabolism, which is slow, are the uptake of pyruvate by the mitochondria and the activity of the pyruvate dehydrogenase complex. According to Wieland et al. (1972) *Neurospora crassa* pyruvate dehydrogenase can be regulated by phosphorylation and dephosphorylation, allowing rapid changes in metabolic activity. However, we could not show any difference in pyruvate dehydrogenase activity or properties between dormant and activated spores (unpublished results). Therefore, attention was focused on pyruvate uptake and metabolism by isolated mitochondria. Although Klingenberg (1970) suggested that pyruvate, like other monocarboxylic acids, needed no carrier for uptake by the mitochondria, there seems now to be general agreement (LaNoue & Schoolwerth, 1979) that, at least for physiologically relevant concentrations, pyruvate uptake by mitochondria is carrier-mediated.

Using homogenization conditions which result in only 50% spore disintegration, we were able to isolate mitochondria in buffers containing 1 M-sucrose. Intact mitochondria could be found in these preparations with the electron microscope. Mitochondria isolated from activated spores decarboxylate [1-14C]pyruvate at a much higher rate than those isolated from dormant spores. However, the activity of the mitochondria from the dormant spores can be brought to the same level by treatments that destroy the integrity of the mitochondrial membrane (the detergent Triton X-100 or resuspension in buffer without osmotic stabilizer). Therefore, the low activity of mitochondria from dormant spores is likely to be caused by a limiting capacity for pyruvate uptake. No such limitation is found in mitochondria from activated spores, since neither of the above treatments results in a significant increase in activity.

The phenomena described start at temperatures inducing germination of the spores, but the increase in mitochondrial activity with temperature continues somewhat further than the increase in germination. Nevertheless, there is a good correlation between the onset of germination triggering and the increase in mitochondrial activity. Therefore, we propose the mitochondrial membrane, and more specifically the mitochondrial pyruvate carrier, as a target for heat activation in *P. blakesleeanus* spores. This is in agreement with the data of Thevelein et al. (1979) who proposed, on the basis of thermodynamic evidence, a protein conformational change as the trigger mechanism in heat activation of *P. blakesleeanus* spores. Since thiol reagents block mitochondrial pyruvate transport (Papa & Paradies, 1974), free thiol groups might be involved in regulation. This could be the mechanism whereby the reducing substances sodium hydrosulphite, sodium hydrogen sulphite and sodium metabisulphite (Van Assche et al., 1978) exert their activating effect on the spores.

In addition, the electron microscopical data of Pambor (1979) point to the mitochondria as the target of heat activation in *P. blakesleeanus*. Indeed, 30 min after activation the characteristic lobate structure of dormant mitochondria disappears and considerable swelling of the mitochondria occurs. Also Cotter (1973) and Cotter & George (1975), working with *Dictyostelium discoideum* spores, suggested, from more circumstantial evidence, that the mitochondria were the target for heat activation. Since spore activation generally involves large increases in respiration, the involvement of the mitochondria in this process could also prove to be a general phenomenon.

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**REFERENCES**


Phycomyces mitochondrial pyruvate metabolism


