Respiratory pathways and oxygen toxicity in 
*Phanerochaete chrysosporium*

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Abstract

*Phanerochaete chrysosporium* maintained on glucose as the carbon source contained severely impaired mitochondria that were characterised by the loss of both succinate dehydrogenase and cytochrome oxidase activities. These cells maintained a constant value for energy charge using anaerobic metabolism. Cells with these properties express lignin peroxidase when supplied with a pure oxygen atmosphere, which may reflect a response to accumulating reactive oxygen species. Cells maintained on cellulose retained fully functional mitochondria, but expressed lignin peroxidase without being exposed to a pure oxygen atmosphere. In the cells maintained on cellulose, mitochondrial function may be limited by the supply of glucose, leading to the accumulation of reactive oxygen species. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Lignin peroxidase (LiP, EC 1.11.1.7) is an extracellular heme enzyme isolated from *Phanerochaete chrysosporium* [1]. The enzyme develops a high oxidising potential with H$_2$O$_2$ to catalyse the oxidation of non-phenolic aromatic compounds [2], including xenobiotics and polymeric lignin [3,4]. Production of LiP is limited by demanding culture conditions. For example, liquid cultures of *P. chrysosporium* supplied with glucose as the carbon source need to be exposed to a pure oxygen atmosphere and starved before the enzyme is expressed [5–11].

In a liquid medium with glucose, fungal hyphae of *P. chrysosporium* aggregate to form pellets and accumulate extracellular polysaccharide. Exposing these cultures to a pure oxygen atmosphere has been proposed to overcome limited diffusion of oxygen into the fungal hyphae [10,12,13]. However, exposing cultures to a pure oxygen atmosphere is fungitoxic when the amount of polysaccharide normally accumulated has been limited by decreasing the initial concentration of glucose supplied [7].

Peroxidases may play an important role in reducing the toxicity of oxygen by reducing H$_2$O$_2$ to water. In the light of its appearance in response to exposing cultures to a pure oxygen atmosphere, LiP may be synthesised as a mechanism for reducing oxidant stress. However, there have been surprisingly few studies of oxygen metabolism coupled to the events leading to LiP expression.

Intracellular oxygen levels are normally maintained at a low relative concentration (0.1 W M, see [14]) by the activity of cytochrome oxidase. However, electrons can also flow to O$_2$ to form water via an alternative, cyanide-resistant, electron transport pathway. Both cytochrome oxidase and the alternative oxidase are present in the mitochondria of young, non-ligninolytic hyphae of *P. chrysosporium* [15].

The purpose of this work was to investigate the rate and possible route of electron transport and oxygen uptake in cultures of *P. chrysosporium*. Also discussed is how these data relate to the cultural conditions that result in the synthesis of LiP.

2. Materials and methods

*P. chrysosporium*, strain BKM-1767, ATCC 24725, was grown in liquid culture at 37°C on a rotary shaker (130 rpm, 2.5-cm cycle) under conditions of non-limiting nutrient nitrogen (20 mM NH$_4$) according to [8] with either
0.4% (w/v) glucose or 0.4% (w/v) Avicel PH101 (Fluka Chemika). Erlenmeyer flasks (2 l) with foam stoppers containing 600 ml of medium were inoculated with 2×10^7 conidiospores of P. chrysosporium. Hyphae from individual flasks during growth were filtered to provide samples of pellets for all measurements except the rate of O_2 consumption. For these, triplicate 10-ml aliquots containing 30-50 pellets were sampled from five flasks, filtered and the pellets together with 1 ml culture medium transferred to the chamber of an O_2 electrode (Rank Broth. Bottisham, Cambridge, UK).

2.1. Isolation of mitochondria

The method used was based on that described previously [16] except that hyphae were disrupted by grinding 50 g (wet weight) of pellets in 500 ml of grinding medium for 4×10 s in an Ultra-Turrax, with 1 min pauses between each 10 s. However, attempts to purify the final preparation on Percoll gradients did not yield a discrete band of mitochondria as found in [16] and washed mitochondria were used in this study.

2.2. Analytical determinations

Extracellular glucose was determined by the Trinder colourimetric method [17]. The oxidation of succinate was measured as O_2 consumption at 25°C in an O_2 electrode (Rank Broth. Bottisham, Cambridge, UK) in a total volume of 1 ml. The assay medium contained 0.25 M sucrose, 10 mM TES, 2 mM MgCl_2, 5 mM potassium phosphate buffer (pH 7.2) and 0.3% (w/v) bovine serum albumin (BSA) (fraction 5). Additions (final concentration) made during measurements were 1 mM succinate, 1 mM (salicylbenzohydroxamic acid) SHAM, 2 mM KCN and 0.25 mM ADP. Mitochondrial protein was solubilised using 5% (v/v) deoxycholate and measured by the method of [18] using BSA as a standard. The cytochromes were determined using a split beam scanning spectrophotometer (Aminco DW2). Adenosine nucleotide extractions were performed according to [19], after disrupting fungal cell walls under liquid N in a mortar with pestle. Estimation of ADP and AMP concentrations was made by enzyme-coupled reactions, using a Perkin Elmer 555 spectrophotometer to detect the oxidation of NADH. ADP was measured by combinations of pyruvate kinase and lactate dehydrogenase. AMP was converted to ADP with myokinase and ATP. Each cuvette contained 500 μl of 0.5 M triethanolamine-HCl (pH 7.6), 4 mM MgSO_4, 6 mM glycercate-3-phosphate, 100 μl NADH (2 mg ml^{-1}) and 200 μl sample. Succinate dehydrogenase was assayed according to [20]. Safranin was used as a spectrophotometric probe to assess the presence of a membrane potential [21]. The enzymic activity of LiP was determined as described by [22]. One unit (U) of activity is defined as the amount of enzyme catalysing the production of 1 μmol of veratraldehyde per minute.

3. Results

Pellets of mycelia were sampled during growth in 0.4% (w/v) glucose (initial concentration) under conditions that would normally favour the expression of LiP if the cultures had been exposed to an atmosphere of pure oxygen when the glucose became exhausted [8,9]. Data in Fig. 1A show that as the supply of glucose became depleted (day 4; see Fig. 1A), the rate of oxygen uptake decreased markedly and paralleled a decline in the level of succinate dehydrogenase activity. However, the adenylate energy charge remained constant throughout this period. The decrease in respiratory rate could be caused by the lack of suitable substrate or by limited penetration of oxygen through accumulating extracellular polysaccharides [12,13]. However, the loss of succinate dehydrogenase activity implied a degradation of mitochondrial function. The metabolic competence of the mitochondria was therefore measured using mitochondria isolated from pellets

![Fig. 1. Kinetics of depletion of extracellular glucose (A) (●) or cellulose (B) (■); rate of O_2 uptake ×10, expressed per pellet (●); content of succinate dehydrogenase (SDH) activity ×10, expressed per pellet (△); adenylate energy charge (▲) and LiP activity (▲) in cultures of P. chrysosporium maintained with either 0.4% w/v glucose (initial concentration) (A) or with cellulose (0.4%) w/v initial concentration (B). Each point represents the mean of at least 3–5 measurements ± S.D.](image-url)
sampled on the 4th day of growth, for which low relative rates of oxygen uptake but high adenylate energy charge was recorded (Fig. 1A). Succinate was used as the electron donor to restrict measurements to the mitochondrial fraction. The maximum electron flow through the terminal oxidases was determined by adding either cyanide to inhibit cytochrome oxidase or SHAM to inhibit the alternative oxidase.

The data in Table 1a show that the overall rate of succinate oxidation was low compared to values recorded in [15] for hyphae grown under conditions of adequate substrate (277 nmol O$_2$ min$^{-1}$ mg$^{-1}$ of protein). Furthermore virtually no respiratory control was evident when ADP was supplied to the preparation. Also, succinate oxidation was not inhibited by the addition of cyanide, which implied that the alternative oxidase was sufficiently active to mediate all of the electron flux from succinate to O$_2$. The addition of 1 mM SHAM to the preparation after cyanide resulted in a 61% inhibition of the rate of respiration (Table 1a) and confirmed that the rate of succinate oxidation was substantially mediated by the alternative oxidase. When the sequence of addition of respiratory inhibitors was reversed (Table 1b) and SHAM was introduced before cyanide, the rate of respiration was only partially inhibited. Subsequent addition of cyanide caused further inhibition, and indicated that cytochrome oxidase was present, but only at a low level. There was a high level of residual respiration resistant to both cyanide and SHAM, which could originate from the auto-oxidation of reduced flavoproteins or ubiquinone. This would be consistent with impaired mitochondrial function.

The respiratory metabolism of cultures maintained on cellulose was also examined. In these cultures, the rate of supply of glucose is regulated by the hydrolytic action of cellulases [23] and LiP is synthesised without requiring the cultures to be exposed to a pure oxygen atmosphere or starved of cellulose ([24]; see Fig. 1B). By the 4th day of growth, the rate of O$_2$ consumption (0.05 ± 0.005 S.D. on measurements from five flasks) nmol min$^{-1}$ pellet$^{-1}$ was significantly faster than the rate recorded for cultures maintained on glucose (0.02 ± 0.002 nmol min$^{-1}$ pellet$^{-1}$) (compare Fig. 1A and B). Data in Table 2a show that the respiratory capacity of mitochondria from these cultures was approximately five-fold greater than that obtained for mitochondria from the glucose-grown cultures. The mitochondria showed a level of respiratory control of 2.4, in contrast to the lack of respiratory control for mitochondria from the glucose-grown cultures. Cyanide (2 mM) caused a 77% inhibition of oxygen consumption, implying an active cytochrome oxidase system. The subsequent addition of SHAM showed the presence of a significant level of alternative oxidase and a residual rate considerably lower than that found for glucose-grown hyphae. The addition of SHAM before cyanide, to inhibit the alternative oxidase (Table 2b), resulted in a much greater inhibition of oxygen consumption than was anticipated from the level of the alternative oxidase derived from Table 2a. Subsequent addition of cyanide reduced the rate of respiration to a residual rate similar to that obtained in Table 2a.

### Table 1

<table>
<thead>
<tr>
<th>Sequence of additions</th>
<th>nmol O$_2$ min$^{-1}$ mg$^{-1}$ of protein (mean ± S.D., n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>15.4 ± 3.8</td>
</tr>
<tr>
<td>+ADP (0.25 mM)</td>
<td>16.7 ± 2.1</td>
</tr>
<tr>
<td>+KCN (2 mM)</td>
<td>16.7 ± 2.1</td>
</tr>
<tr>
<td>+SHAM (1 mM)</td>
<td>6.5 ± 2</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>19.2 ± 0</td>
</tr>
<tr>
<td>+ADP (0.25 mM)</td>
<td>19.2 ± 0</td>
</tr>
<tr>
<td>+SHAM (1 mM)</td>
<td>10.15 ± 1.9</td>
</tr>
<tr>
<td>+KCN (2 mM)</td>
<td>6.3 ± 0.2</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>nmol O$_2$ min$^{-1}$ mg$^{-1}$ of protein (mean ± S.D., n = 3)</th>
</tr>
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<tbody>
<tr>
<td>(a)</td>
</tr>
<tr>
<td>Succinate</td>
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<tr>
<td>+ADP (0.25 mM)</td>
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<td>+KCN (2 mM)</td>
</tr>
<tr>
<td>+SHAM (1 mM)</td>
</tr>
<tr>
<td>(b)</td>
</tr>
<tr>
<td>Succinate</td>
</tr>
<tr>
<td>+ADP (0.25 mM)</td>
</tr>
<tr>
<td>+SHAM (1 mM)</td>
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<tr>
<td>+KCN (2 mM)</td>
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Fig. 2. The reduced minus oxidised cytochrome spectrum from mitochondria extracted from 4 day old pellets of *P. chrysosporium* maintained with either glucose (a) or cellulose (b). Between 1.5 and 2 mg of mitochondrial protein were suspended in 1.0 ml of oxygen electrode assay medium. The sample was then reduced with 1–2 mg of solid dithionite. The wavelength pairs and extinction coefficients were those published in [27].
The concentration of cytochromes in the preparation was the region of 602 nm, consistent with their degeneration. The much stronger absorption peak at 602 nm indicated a cytochrome spectrum for mitochondria from the cellulose-protein, respectively. By contrast, examination of the cytochrome oxidase per mg of protein, respectively. The presence of the higher level of cytochrome oxidase and respiratory control for mitochondria from the cellulose-grown hyphae (Fig. 2b) confirmed their superior quality. The high level of cytochrome oxidase and measurement showed that these mitochondria contained 0.36 nmol of cytochrome c, 0.35 nmol cytochrome b and 0.35 nmol cytochrome oxidase per mg of protein, respectively. The presence of the higher level of cytochrome oxidase and respiratory control for mitochondria from the cellulose-grown hyphae is consistent with the view that these fungi have fully functional mitochondria. This was confirmed by measuring the energy-conserving characteristics of the cellulose-grown mitochondria, using the spectral shift in safranin. Fig. 3a shows the energisation of the mitochondria upon the addition of succinate, which was subsequently collapsed when carbonylcyanide-p-trifluoro-methoxyphenylhyrazone (FCCP) was added. These data show that the mitochondria were able to sustain a membrane potential. The trace in Fig. 3b showed that cyanide also resulted in a collapse of the membrane potential implying that the cyanide resistant alternative oxidase could not sustain an electrochemical proton gradient across the membrane system. Fig. 3c shows that the addition of SHAM caused a slight increase in energisation, which was subsequently collapsed by the addition of cyanide. This confirmed that the energisation of the mitochondria was mediated only by electron transport via cytochrome oxidase; it also explained the lack of respiratory control observed in the mitochondrial preparation obtained from pellets maintained on glucose.

From these data we can conclude that mitochondrial function became impaired in cultures maintained on glucose, in line with the decline in rate of oxygen uptake that was measured, which is consistent with the increasingly reduced nature of intracellular compartments prior to lignin peroxidase expression [25]. In turn, anaerobic metabolism must represent the main route for ATP synthesis to maintain the constant energy charge observed. Consequently, it is likely that exposing these cells, which have a reduced capacity to reduce O2 to water, to hyperbaric oxygen could give rise to a hyperoxidant state and trigger LiP synthesis as a means to reduce partially reduced species of O2 namely H2O2, to water.

Some comment is necessary concerning the appearance of LiP in the cellulose-maintained cultures since the data indicated that their mitochondrial function was not impaired. A hyperoxidant state will arise when the availability of O2 exceeds the supply of reducing equivalents from carbon metabolism [26]. In these cultures the supply of glucose to the hyphae will be limited by the rate of cellulose hydrolysis by extracellular cellulases; this limited the synthesis of extracellular polysaccharide, and decreased the permeability barrier to oxygen (see Fig. 1B). Mitochondrial cytochrome oxidase, a major route for the reduction of O2 to water, remained functional in these cultures. Probably, the rate of O2 reduction via cytochrome oxidase was limited by the supply of reducing equivalents obtained from carbon metabolism, leading to the development of a hyperoxidant state and expression of LiP.

References


