Short Communication

Cytochrome Oxidase Activity in Cell-free Preparations from Blue-Green Algae1,2

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The chlorophyll-containing lamellar structures isolated from Anabaena variabilis have the ability to oxidize reduced mammalian cytochrome c in the dark. This activity is oxygen dependent and heat labile. As with other cytochrome oxidase preparations, activity is stimulated by detergents and is sensitive to the ionic strength of the assay solution. The cytochrome oxidase activity is not inhibited by azide, is slightly inhibited by carbon monoxide, and is readily inhibited by potassium cyanide. This sensitivity to inhibition by cyanide distinguishes the cytochrome oxidase from cytochrome photo-oxidase which is cyanide insensitive (6).

There are several studies indicating the feeble respiratory activity of blue-green algae (2-5, 9, 16, 20, 21). Cell-free preparations from blue-green algae have been found to catalyze respiratory chain oxidations (3, 4, 8, 12, 20). Whereas reduced cytochrome c photooxidation is regularly observed on illumination of particulate preparations from blue-green algae (7, 14), a “dark” cytochrome oxidase such as would participate in a respiratory chain has not revealed itself to all experimenters. Biggins (4) reported cytochrome oxidase in preparations of Anacystis nidulans and Phormidium luridum; yet Horton (8) could not observe cytochrome oxidase activity in preparations from Anabaena variabilis, A. nidulans or Leuchothrix which were active in NADH oxidation. Using “colorless” species of blue-green algae Webster and Hackett found, as did Horton, that particulate preparations exhibited NADH oxidase but not cytochrome oxidase (20).

This paper reports experiments which describe a cytochrome oxidase activity in particulate preparations from A. variabilis.

MATERIALS AND METHODS

A. variabilis and Plectonema boreyanum were grown at 35 and 25 C respectively in Kratz and Myers medium A (10) with vigorous aeration and at a tungsten light intensity of 3 x 104 ergs/cm2-sec. Photosynthetic lamellae were isolated by the procedure of Lee et al. (13). Chlorophyll measurements were as previously described (18), and protein content was estimated from micro Kjeldahl nitrogen analysis of the isolated lamellae. Cytochrome c oxidase activity was measured with an Eppendorf 1100 colorimeter with appropriate filters and a strip chart recorder. The reaction mixture could be cross illuminated with an actinic beam (680 nm Baird atomic narrow band pass filter, 5 x 104 erg/cm2-sec) to elicit cytochrome c photooxidase activity.

Horse heart cytochrome c was purchased from Sigma Chemical Company. A stock solution containing twenty mg per ml of cytochrome c was reduced with excess sodium borohydride, then dialyzed before use. A difference in millimolar extinction coefficients of oxidized and reduced cytochrome c at 550 nm of 18.8 was used.

The standard reaction mixture used in all experiments, unless otherwise noted, contained 2 mg of reduced cytochrome c, 300 μmoles of sodium potassium phosphate buffer, pH 7.8, 0.03% SDS and lamellae containing 30 μg of chlorophyll in a total volume of 3 ml. The reaction mixture was stirred continuously during all measurements.

Oxygen saturation data were obtained by bubbling pure O2 through the reaction vessel to achieve an increase in O2 tension as measured with a YSI oxygen electrode. This electrode was also used to measure O2 consumption associated with the cytochrome oxidation.

Reaction rates are expressed as μmoles ferrocyanochrome c oxidized per mg chlorophyll per hour. This is a convenient expression since the amount of algal lamellae is most easily established by a chlorophyll measurement. The A. variabilis cells used in these studies contain 1.75 to 2% chlorophyll and the lamellae preparations contain 15 to 17 mg protein per mg chlorophyll.

RESULTS

Figure 1 shows results from a typical assay of cytochrome oxidase activity. On mixing reduced cytochrome c with the lamellae, there is a decrease in absorbance at 550 nm which is greatly accelerated by a subsequent addition of the detergent SDS. If the experiment is repeated with illumination of the reaction mixture, then the detergent-activated cytochrome c photo-oxidase is observed as seen in Figure 1. Both dark oxidase and photooxidase activities are associated with the chlorophyll containing particles since washing these particles by sucrose density gradient centrifugation (19) did not separate the oxidase activities from the green particles although the treatment removes all phycocyanin, ferredoxin and other readily solubilized components. Cytochrome oxidase has been reported in spinach chloroplast preparations (15), and we could readily repeat these experiments. However, the spinach cytochrome oxidase could be completely removed from the chloroplasts by density gradient centrifugation. Since the spinach

1 Abbreviations: SDS: sodium dodecyl sulfate; CTAB: cetyl trimonium bromide; PCMB: para chloro mercury benzoate; CCCP: p-chlorocarboxyl cyanide phenylhydrazone; HOQNO: hydroxyquinoline-N-oxide.

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cytochrome oxidase moved with succinic dehydrogenase in the sucrose gradients, it appears to be associated with mitochondrial contaminants of the spinach chloroplasts.

The oxidation of cytochrome $c$ in the presence of 0.03% SDS is proportional to the amount of lamellae. The nonlinearity at low lamellae concentrations may be the result of inhibition by SDS since detergent stimulation or inhibition of this reaction appears to be dependent on the ratio of enzyme to detergent. This is indicated in Figure 2, which shows the response of cytochrome oxidase to varying amounts of SDS. Table I indicates the relative effectiveness of various detergents in activating cytochrome oxidase. The concentrations cited are the ones giving maximum response when the detergent was tested over a large range of concentrations. The cationic detergent CTAB inhibited the cytochrome oxidase activity at all concentrations tested.

The cytochrome $c$ activity of these particles is saturated by 10 mg reduced cytochrome $c$ per ml, which is about twice as much reduced cytochrome $c$ as that needed to saturate the cytochrome $c$ photo-oxidase (14). Oxygen is the other substrate for this reaction. The effects of variation in concentration of oxygen on both cytochrome oxidase activities are described by Figure 3. Although cytochrome $c$ photo-oxidase responds to increasing $O_2$ concentrations, the dark oxidase activity is not much altered by increasing $O_2$ concentrations above 20% of saturation. If the reaction was attempted in an anaerobic cuvette which had been flushed with $N_2$ and evacuated three times, then no cytochrome oxidation could be observed in either dark or light.

Cytochrome oxidase activity is little influenced in the range between pH 6 and pH 8. Like mammalian cytochrome oxidase, the activity of Anabaena lamellae is sensitive to the ionic strength of the environment. A phosphate buffer concentration of 20 mM is optimal for this activity. A variety of salts gave similar effects.

Table I. Cytochrome Oxidase Response to Detergents

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Rate (µmoles cytochrome $c$ oxidized/mg chl-hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.1</td>
</tr>
<tr>
<td>0.03% SDS</td>
<td>20.8</td>
</tr>
<tr>
<td>0.01% Triton X-100</td>
<td>6.1</td>
</tr>
<tr>
<td>0.12% Tween 20</td>
<td>9.3</td>
</tr>
<tr>
<td>0.50% Tween 80</td>
<td>4.5</td>
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</tbody>
</table>

Table II. Inhibition of Cytochrome Oxidase Activity

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Rate (µmoles cytochrome $c$ oxidized/mg chl-hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27.2</td>
</tr>
<tr>
<td>Heated lamellae</td>
<td>0.0</td>
</tr>
<tr>
<td>CO</td>
<td>21.6</td>
</tr>
<tr>
<td>0.3 mM Rotenone</td>
<td>10.6</td>
</tr>
<tr>
<td>4.0 mM NH$_2$OH</td>
<td>8.3</td>
</tr>
<tr>
<td>1.0 mM KCN</td>
<td>9.0</td>
</tr>
<tr>
<td>0.06 mM PCMB</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Fig. 1. Oxidation of reduced cytochrome $c$ by *A. variabilis* lamellae.

Fig. 2. Effect of SDS on cytochrome oxidase activity.

Fig. 3. Effect of oxygen concentration on cytochrome oxidase activity. The data described by the circles is cytochrome oxidase activity, whereas that described by the triangles is cytochrome $c$ photo-oxidase activity.
Table II illustrates the inhibition of the cytochrome oxidase. Heating the lamellae above 75 C for 3 min abolished activity. Carbon monoxide had only a slight effect on the reaction although it completely inhibited a mammalian cytochrome oxidase control. Rotenone, hydroxylamine, KCN, and PCMB all inhibit the cytochrome oxidase. When tested to the limit of their solubility, the following compounds did not inhibit *Anabaena* cytochrome oxidase: antimycin A, CCCP, HQQNO, diethylthiocarbamate, bathophenanthroline, and sodium azide.

**DISCUSSION**

It is important to compare the activities reported in this study with other published data. Kratz and Myers (9), Cheung and Gibbs (5), and Biggins (2, 4) have reported similar values for the endogenous respiration of intact blue-green algae. These values from the literature (4) indicate a rate of 48 μmoles cytochrome c oxidized per mg chlorophyll per hour. If all of this respiration proceeded through cytochrome oxidase, one would expect a rate of 48 μmoles cytochrome c oxidized per mg chlorophyll per hour, twice the rate routinely observed in these experiments with detergent present. We have occasionally observed detergent-stimulated rates equivalent to the whole cell respiratory rate. Lamellae preparations from *P. boryanum* showed a rate of 75 μmoles cytochrome c oxidized per mg chlorophyll per hour in the presence of SDS. To make the match between the in vivo and the in vitro rates, one must assume that detergent elicits a level of activity which reflects the normal rate of function within the cell.

Detergent activation of mammalian cytochrome oxidase is routinely observed (16). Cytochrome c photooxidase activity in *A. variabilis* requires detergent activation but Tween 20, which is a rather poor activator of the dark cytochrome oxidase activity, gives maximal activation of the cytochrome c photooxidase (14). In both cases, the detergent probably facilitates access of reduced cytochrome c to the oxidase site within the particle. Stimulation by salts of both cytochrome oxidase observed here and of cytochrome photo-oxidase (15) probably is due to dissociation of ionic interactions between the cytochrome and the photosynthetic membranes (1).

The location of a potential respiratory enzyme system in the photosynthetic lamellae is noteworthy. It is entirely possible that the cytochrome oxidase activity described here is not a respiratory enzyme and is an expression of some other type of oxidation-reduction activity. However, blue-green algae contain no clearly visible respiratory structure and may well have organized their rather feeble respiratory components on photosynthetic membranes. Horton’s report on NADH oxidase (8) and Biggins’ more extensive studies of the respiratory activity in cell-free preparations from blue-green algae (4) indicate possible localization of these activities on the photosynthetic membranes. Although both the cytochrome oxidase and the cytochrome photooxidase activities are stimulated by detergents, it seems that they are distinct and separate enzyme systems. The cytochrome photooxidase reaction is not inhibited by cyanide as was first shown by Nieman and Vennesland (15) using spinach chloroplasts and as confirmed in our laboratory using blue-green algae lamellae. The photooxidase activity can be greatly increased by addition of viologen dyes and exogenous plastocyanin (14), neither of which stimulate the cytochrome oxidase.

The affinity for oxygen by these reactions is another distinguishing characteristic. The photooxidase responds to increasing oxygen tensions well above the level of O₂ saturation for the cytochrome oxidase. Saturation of the oxygen requiring step at normal oxygen tension argues against a chemical autodissociation of a reduced electron carrier. Measurements with the oxygen electrode indicated a stoichiometry of one atom of oxygen consumed for every two molecules of cytochrome c oxidized. We attempted to detect H₂O₂ as a product of the cytochrome oxidase activity using a sensitive enzymatic assay (11), but found that H₂O₂ appeared in these reactions at a rate of less than 10% of that which would be expected if all of the cytochrome oxidation resulted in the reduction of oxygen to H₂O₂.

Attempts at inhibiting the respiration in blue-green algae have given a variety of results. Cyanide is generally found to inhibit the respiration of both intact cells (4, 20, 21) and of cell-free respiratory activities (3, 4, 8) as reported here. Webster and Frenkel (21) had reported inhibition of respiration of *A. variabilis* by azide, but this inhibition was not seen in the experiments of Webster and Hackett on *Vitrioscillia* (20), or in the cell-free measurements of Horton (8) or in our experiments. Rotenone blocked the NADH oxidase of cell-free preparations from three blue-green algae (8), although at a considerably lower concentration (60 μM) than that (0.3 mM) needed to suppress cytochrome oxidase in our experiments. Carbon monoxide has proved an effective inhibitor of both whole cell and cell-free respiratory activities of blue-green algae (3, 4, 20). The inability to observe carbon monoxide inhibition of cytochrome oxidase in our experiments has no obvious explanation.

**LITERATURE CITED**


7. **Fujiya, Y. and J. Myers. 1965. Cytochrome c redox reactions induced by photochemical system 1 in sonicated preparations of *Anabaena cylindrica*. Arch. Biochem. Biophys. 113: 790-797.**


