Proton Pump Coupled to Cytochrome c Oxidase in the Cyanobacterium *Anacystis nidulans*

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Intact spheroplasts of the cyanobacterium *Anacystis nidulans* were found to oxidize various exogenous c-type cytochromes with concomitant proton extrusion. In the coupled state, $H^+|e^-$ stoichiometries close to 1 were measured, regardless of absolute reaction rates. It is concluded that the proton translocation observed is an intrinsic property of the cytoplasmic membrane-bound cytochrome c oxidase of *A. nidulans*.

According to the chemiosmotic theory (11, 12), energy conservation (ATP synthesis) through electron transport in biological membranes depends on a proton electrochemical gradient across the membrane. In mitochondrial oxidative phosphorylation, one of the mechanisms by which the gradient is formed appears to be the direct conformational proton pumping by the cytochrome oxidase (EC 1.9.3.1) (7, 23). The activity is thought to be mainly associated with a proton channel in subunit III of the enzyme (1). Among bacteria, the situation is less clear; only a few species were shown to contain $aa_3$-type terminal oxidases similar to mitochondria (9), and preliminary evidence for proton pumping by the cytochrome oxidase has been obtained so far only with *Paracoccus denitrificans* (22). Yet the isolated *Paracoccus* oxidase was found to consist of two subunits only (10), the two polypeptides being homologous to subunits I and II of the mitochondrial enzyme (1). Terminal oxidases from cyanobacteria have not yet been obtained in a purified state. However, in crude membrane preparations (comprising both cytoplasmic and thylakoid membranes), they were shown spectrophotometrically to be of $aa_3$ type (6, 14, 15). Cyanobacteria-like organisms probably were the first that produced free oxygen through plant-type photosynthesis and might have been among the first to be capable of aerobic respiration (2–4). Aerobic respiration, in turn, was needed for the evolution of higher (eucaryotic) organisms (3). In this paper, it is shown that, in addition to several other "mitochondria-like" features of the cyanobacterial cytochrome c oxidase previously reported (6, 13–15, 18, 19), the enzyme present in the cytoplasmic membrane of *Anacystis nidulans* apparently can act as a proton pump. A preliminary account of these findings will be published (Peschek et al. *in G. C. Papageorgiou and L. Packer, ed., Photosynthetic procaryotes: cell differentiation and function, in press*). Axenic cultures of *A. nidulans* (*Synechococcus* sp., strain L-1402-1, Culture Collection of Algae, Göttingen, Federal Republic of Germany) were grown at 38°C and 1,000 W·m$^{-2}$ as described previously (17). Cells were harvested during late logarithmic growth and washed twice with 10 mM sodium-EDTA adjusted to pH 8 with a few crystals of K$_2$HPO$_4$. Spheroplasts were prepared in a medium containing 0.4 M mannitol, 30 mM KCl, 25 mM N-2-hydroxethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-K$_2$HPO$_4$ buffer (pH 6.9), and 0.5% (wt/vol) lysozyme. After incubation at 37°C for 7 h, almost 100% spheroplasts had formed; they were centrifuged (900 $\times$ g for 20 min), washed twice with lysozyme-free medium, and resuspended in the assay medium (0.4 M mannitol, 20 mM KSCN, 30 mM KCl, 0.1 mM sodium-EDTA, 0.5 mM N-ethylmaleimide, 1 mM HEPES-KOH, 50 $\mu$g of carbonic anhydrase per ml, 7.5 $\mu$g of oligomycin per ml, 0.8 $\mu$g of valinomycin per ml [final pH 7.2]). The spheroplasts remained intact, as seen from a complete lack of phycocyanin and ferredoxin in the supernatants (not shown). Integrity of the spheroplasts was a prerequisite for assuring that the exogenous c-type cytochromes were oxidized on the cytoplasmic membrane and not through thylakoid-bound cytochrome oxidase.

Oxidation of chemically reduced and dialyzed c-type cytochromes (6, 14) catalyzed by the spheroplasts was followed at the appropriate wavelength pairs in temperature-controlled cuvettes containing 2.5 ml of suspension by using a Shimadzu dual wavelength spectrophotometer, model UV-300, assuming a differential absorb-
TABLE 1. Proton ejection coupled to the oxidation of c-type cytochromes by intact spheroplasts of _A. nidulans_ a

<table>
<thead>
<tr>
<th>Source of cytochrome</th>
<th>Oxidation of cytochrome</th>
<th>Proton translocation</th>
<th>H⁺/e⁻ stoichiometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse heart</td>
<td>7.5 ± 1.5</td>
<td>6.8 ± 2.0</td>
<td>0.91 (7)</td>
</tr>
<tr>
<td>Tuna</td>
<td>4.4 ± 1.0</td>
<td>4.2 ± 1.2</td>
<td>0.83 (4)</td>
</tr>
<tr>
<td><em>Candida krusei</em></td>
<td>2.8 ± 0.5</td>
<td>2.9 ± 0.8</td>
<td>1.04 (5)</td>
</tr>
<tr>
<td><em>Saccharomyces oviformis</em></td>
<td>2.4 ± 0.5</td>
<td>2.6 ± 0.7</td>
<td>1.05 (2)</td>
</tr>
<tr>
<td><em>Rhodocyclus purpureus</em></td>
<td>6.7 ± 1.3</td>
<td>5.6 ± 1.5</td>
<td>0.83 (4)</td>
</tr>
<tr>
<td><em>Rhodopseudomonas palustris</em></td>
<td>5.8 ± 1.0</td>
<td>5.1 ± 1.5</td>
<td>0.88 (3)</td>
</tr>
</tbody>
</table>

a Oxidation of the cytochromes was followed by dual wavelength spectrophotometry. Proton ejection was measured with a pH electrode. Initial rates are expressed as nanomoles of cytochrome c or H⁺ per minute per milligram of protein. Numbers in parentheses indicate the numbers of parallel experiments. For experimental details, see the text.

...ution coefficient of $\epsilon = 20 \text{mM}^{-1} \text{cm}^{-1}$ throughout (6). Alternatively, oxygen uptake in the presence of reduced cytochrome c was measured with a Clark type electrode (14, 17). pH changes in the suspension medium were followed either directly with a Philips digital pH meter, model PW 9409, connected to a Görtz recorder, or spectrophotometrically at 560 – 580 nm, with 60 μM phenol red as a pH-indicating dye (7, 23). Cytochrome c oxidation (or oxygen uptake) and proton movements were measured simultaneously with one batch of spheroplasts (1.9 mg of protein per ml). The cuvette of the spectrophotometer and the sample compartments (2.5 ml) of pH electrode and oxygen electrode were connected to the same thermostat (30°C). Resulting H⁺/e⁻ ratios were independent, within experimental error (Table 1) of the type of measurement employed. Inhibitors were added 3 min before starting the measurements. Protein was determined according to the method of Lowry et al. (8). No cytochrome c oxidation or H⁺ extrusion could be measured with anaerobic spheroplast suspensions assayed in argon-flushed Thunberg cuvettes (control experiments not shown).

H⁺/e⁻ stoichiometries were calculated from initial rates of cytochrome c oxidation or cytochrome c-supported oxygen uptake and H⁺ ejection. They were close to the supposed theoretical value of 1.0 (23) when reduced cytochrome c was the only electron donor present (Fig. 1 and Table 1). However, in the presence of both ascorbate and cytochrome c, the apparent H⁺/e⁻ ratio was raised from 0.89 (Fig. 1) to 1.33 (Fig. 2), i.e., by about 50%. At pH 7, ascorbate releases 1H⁺ per 2e⁻ upon oxidation (22, 23), and the scalar proton adds to the 2H⁺/2e⁻ from vectorial proton translocation by the cytochrome oxidase, as observed (compare Fig. 1 and 2).

The uncoupler carbonyl cyanide _m-_chlorophenylhydrazone (CCCP) abolished any initial acidification phase linked to cytochrome c oxid...
functioning cytochrome observed, the latter aerobic spheroplasts, the same (or H\(^+\)) per minute per milligram of protein and are given adjacent to the curves. Upward deflection in (B) indicates acidification of the medium.

dation; instead, alkalization of the medium was observed (Fig. 1 and 2), which is attributed to the proton-consuming reduction of oxygen within the cell (O\(_2\) + 4e\(^-\) + 4H\(^+\) = 2H\(_2\)O) accompanied by slow leakage of H\(^+\) into the cell (23). Cyanide depressed cytochrome c oxidation and proton translocation to the same extent without affecting H\(^+\)/e\(^-\) stoichiometry (0.82 H\(^+\)/e\(^-\)) (Fig. 3). In contrast, dicyclohexylcarbodiimide (DCCD), an ATPase inhibitor supposed to plug certain proton channels across membranes (21), consistently inhibited proton translocation more strongly than cytochrome c oxidation (compare references 5 and 21), as reflected by a significantly lowered H\(^+\)/e\(^-\) ratio (0.37 H\(^+\)/e\(^-\)) (Fig. 3). This ratio remained constant over a range of DCCD concentrations of 5 to 100 \(\mu M\) (results in Fig. 3 given for 50 \(\mu M\) DCCD only), thus probably ruling out a simple uncoupling action of DCCD. Table 1 shows that roughly the same H\(^+\)/e\(^-\) stoichiometry was obtained, regardless of the source of the cytochromes and hence regardless of absolute reaction rates. When reduced cytochrome c was added to anaerobic spheroplasts, no proton extrusion was observed, the latter obviously depending on a functioning cytochrome oxidase. Therefore, the proton translocation observed appears to be an intrinsic property of the cytochrome oxidase. At the same time, the reaction of exogenous cytochrome c with intact spheroplasts in itself supports the view that part of the cytochrome oxidase of A. nidulans is associated with the cytoplasmic membrane (13, 16, 17, 20).

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