Photosynthetic Reactions by Lysed Protoplasts and Particle Preparations from the Blue-Green Alga, Phormidium luridum1, 2

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Summary. Reactions of photosynthetic electron transport and photophosphorylation were studied in preparations from the blue-green alga, Phormidium luridum. Osmotic lysis of protoplasts proved to be a superior technique for the production of cell-free preparations with high enzymatic activity. Such lysed protoplasts sustain high rates of photophosphorylation coupled to the photo-reduction of NADP+ or ferricyanide. P/2e- ratios close to unity were routinely observed. The same preparations, and also those prepared by grinding the cells in solutions containing sucrose or ethylene glycol, are active in cyclic photophosphorylation mediated by phenazine methosulfate or dichlorophenolindophenol. The particles prepared by grinding the cells are, however, inactive in non-cyclic photophosphorylation.

Extensive washing of the membranes with solutions containing sucrose removes the majority of the residual soluble fraction of the algal cell which includes cytochromes C554 and C549, and phycocyanin. Cyclic photophosphorylation activity is unpaired by this treatment, but is abolished when the membranes are washed with very dilute buffers. This activity is restored by the addition of a soluble protein which is not a known redox constituent such as cytochrome C554 or plastocyanin, and may be a coupling factor.

Analysis of the well-washed membranes by low temperature (77°K) difference spectrophotometry reveals the presence of cytochrome b5 and a bound form of cytochrome C554 in proportions similar to that found in higher plant chloroplasts. The concentration of the membrane-bound cytochrome C554, relative to cytochrome b5, is not altered by extensive washing, sonication or treatment with 1% digitonin. This indicates that this cytochrome is an integral component of the cytoplasmic lamellae and we suggest that it is of functional significance. The soluble form of cytochrome C554, which is present in concentrations about 3-fold higher than the bound form, depending upon growth conditions, is not essential for cyclic photophosphorylation. The concentration of cytochrome b5: chlorophyll a was found to be 1:500.

Under the conditions employed, we were unable to detect a bound form of the low potential cytochrome C549.

The blue-green algae are unique in that they are proaryotic but their photosynthetic mechanism is more typical of eucaryotic cells. As is the case in green algae and higher plants, 2 photosystems are necessary and the high potential oxygen evolution mechanism is present (30).

An important difference between the morphology of the photosynthetic apparatus of blue-green algae and the chloroplasts of higher plants is the degree of molecular organization of the constituents. In common with the red algae and cryptomonads, the accessory pigments in the blue-green algae are soluble biliproteins and are readily removed from their location on the chlorophyll-bearing lamellae by aqueous washing (25). Certain soluble cytochromes are present in blue-green algae in high concentrations. Katoh extracted a soluble cytochrome of type c from Tolypothrix tenuis and noted its possible equivalence with the photosynthetic cytochrome f found in the chloroplasts of higher plants (21). This cytochrome, and also a low potential soluble cytochrome type c (549, Anacysis), has been purified and fully characterized by Holton and Myers (18, 19, 20). Thus, as noted by Susor, Duane, and Krogmann (30), the mechanical ease by which these components are removed from the photosynthetic lamellae renders the blue-green algal system particularly amenable to the study of photosynthesis at the biochemical level. It offers, for example, the possibility for the reconstitution of the photosynthetic apparatus using purified components. Hence, studies of the partial reactions

1 This investigation was supported by the National Science Foundation, GB-4046.
2 A preliminary account of this investigation was presented at the 1966 AIBS Meetings, College Park, Maryland.
of photosynthetic electron transport may lead to specific functional assignments for these soluble components.

Available reports concerning the photosynthetic mechanism in blue-green algae indeed confirm that the apparatus is quite susceptible to physical disruption by very mild procedures. For instance, in an investigation of the Hill reaction in *Anacystis nidulans*, Fredricks and Jagendorf found that aqueous extraction of lamellar fragments led to a loss in activity (9). The activity could be restored by the addition of a macromolecular component in the soluble extract. Apart from confirming that the component was not phycoerythrin, the restorative factor was not characterized. Duane, Hohl, and Krogmann (7) were unable to demonstrate photophosphorylation coupled to the photoreduction of NADP+ or ferri-cyanide by preparations from *Anabaena* which were, nevertheless, very active in cyclic photophosphorylation. Extracts of *Anacystis nidulans* were found to be inactive in cyclic and non-cyclic photophosphorylation although the same preparations were capable of electron flow (5). Gerhardt and Trebst (11) have recently reported non-cyclic photophosphorylation by preparations from *Anacystis nidulans* but the observed rates and P/2e− ratios are low.

The present report is concerned with photosynthetic electron transport and photophosphorylation in the blue-green alga *Phormidium luridum*. We have investigated the functional role of the soluble redox components in photophosphorylation, and, in an effort to preserve the coupling of ATP synthesis to electron flow, we have successfully utilized the rupture of metabolically active protoplasts (3) as a means of preparing cell-free extracts.

**Methods**

*Algae and Protoplasts.* *Phormidium luridum* was cultured and protoplasts were isolated by the procedure described in the accompanying report (3). Protoplasts were washed once with either ice-cold mannitol-tris-maleate pH 7.2, mannitol-tris-Cl (pH 7.5), or 0.5 M mannitol titrated to neutrality.

For large scale enzyme preparations, algae were cultured in 5 gallon carboys and rapidly flushed with air. Illumination was provided by a bank of fluorescent tubes. The algae were harvested by continuous flow centrifugation, washed once in metal-free water and frozen. Stocks were stored at −20°C.

**Membrane Preparations.** Cell-free preparations were created by adding the protoplasts to the reaction mixture of interest, thereby causing an 8-fold dilution of mannitol which resulted in cell lysis. Microscopic examination of such reaction mixtures revealed that about 95% cell lysis had occurred indicating that some cells were still protected by cell wall.

Membrane fragments were also prepared by grinding the washed cells with sand. The homogenate was extracted with 10 volumes of either 40% ethylene glycol, 0.05 M tris-Cl (pH 7.5) or 0.3 M sucrose, 0.05 M tris-Cl (pH 7.5). The homogenate was centrifuged at 5000 g to remove sand, whole cells and large fragments and then at 27,000 g for 15 minutes to sediment heavy particles. This supernatant was then centrifuged at 48,000 g for 30 minutes to sediment light particles. Residual chlorophyll in the final supernatant amounted to about 5% of the total chlorophyll in the homogenate. Chlorophyll a was measured spectrophotometrically after extraction with cold 80% acetone using $F_m/662nm = 82$.

**Photophosphorylation.** Photophosphorylation was carried out using an illuminated Warburg apparatus at 25°C. The gas phase and reaction conditions were as indicated. The incorporation of $^{32}$P into ATP was measured in trichloroacetic acid-denatured supernatants by the method of Avron (1).

**Photoreduction.** As a measure of electron flow during non-cyclic photophosphorylation, NADPH was determined spectrophotometrically after the incubation period prior to denaturation of the reaction mixtures with trichloroacetic acid. Ferrocyanide was determined by the procedure of Avron and Shavit (2).

**Spectrophotometry.** Room temperature spectra of solutions were obtained using a Cary Model 15 automatic recording spectrophotometer. Low temperature (liquid $N_2$) and room temperature spectrophotometry of particles was carried out using a more modern version of the split-beam spectrophotometer described by Chance and co-workers (6, 36). A large end-on phototube (Dumont KM2433) was used. For low temperature studies, particles were suspended in 0.4 M sucrose, 0.05 tris-Cl (pH 7.5) to enhance the band intensities (35) and the samples were rapidly frozen in precooled Plexiglass cuvettes of 2 mm light path. During measurements the samples were maintained close to liquid nitrogen temperature in an unsilvered glass Dewar.

**Biochemicals.** NADP+ and ADP were obtained from Boehringer Manheim Corporation, New York; 2,6 dichlorophenol indophenol (DPIP) and phenazine methosulfate (PMS) from Sigma, and $^{32}$P from Squibb, New Brunswick. Dr. R. Goldsby, Central Research Department, DuPont Chemical Company, Wilmington, Delaware kindly provided 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU).

**Protein Preparations.** Crude aqueous extracts of the algae were prepared by pouring a 500 ml cold suspension of cells (100 g wet wt) into 4 volumes of −20°C acetone with vigorous stirring. The resulting slurry was then centrifuged at low speed and the yellow-brown supernatant discarded. The precipitate was homogenized in 1 liter 0.05 M phosphate (pH 7.5), 1.0 M ferrocyanide and the plastocyanin solution was then centrifuged and the blue supernatant retained as the crude extract. The extract was then made 55% saturated with ammonium sulfate at pH 7.5 and, after 30 minutes, was centrifuged. Usually all of the phycocyanin was removed by this step. The 55% saturated solution was then made 100% saturated with ammonium sulfate and stirred overnight in the cold. The pre-
precipitated protein was collected by high speed centrifugation at 48,000 g for 20 minutes and suspended in 10 mM phosphate (pH 7.5). The protein was dialyzed against the same buffer to remove salt. This solution contained ferredoxin, phytoflavin, ferredoxin-NADP" oxidoreductase (NADP" :ferredoxin oxidoreductase, EC 1.6.99.4), cytochrome-C54, cytochrome-C484, plastocyanin and the PMS-cyclic photophosphorylation component.

**Cytochrome C544 and Plastocyanin.** The dialyzed extract was passed through a column, 3 × 20 cm, packed with DEAE-cellulose equilibrated with 10 mM phosphate (pH 7.5). Cytochrome C544 and plastocyanin were not absorbed and this effluent was then made 10 mM in ferri cyanide. This solution was then drained through a column, 1 × 10 cm packed with CM-cellulose. The ferricytochrome C544 was not absorbed whereas the oxidized plastocyanin was retained as a blue band near the top of the column. The column was washed with 10 mM phosphate (pH 7.5), 1.0 mM ferri cyanide and the plastocyanin was then eluted with 25 mM phosphate (pH 7.5). The protein was dialyzed and then stored as a frozen solution at −20°C.

Figure 1 shows an absolute absorption spectrum of Phormidium plastocyanin after oxidation with ferricyanide. It is similar to the plastocyanin of higher plants (23), green algae (12) and *Anabaena* (24) in that there is a broad absorption band in the red with a maximum at 597 nm and complex bands in the ultraviolet.

Cytochrome C544 was precipitated from the eluate by ammonium sulfate and stored in saturated ammonium sulfate solution at 4°C. Figure 2 shows room temperature and liquid nitrogen absolute spectra of the reduced form of this cytochrome. At room temperature our observed absorption maxima agree very well with those reported for the *Anacystis* cytochrome by Holton and Myers (18, 19, 20) and the *Anabaena* cytochrome by Susor and Kroghmann (31). At liquid nitrogen temperature the splitting of the absorbance maximum was accompanied by a blue shift of the absorption maximum. This behavior is similar to that described by Hill and Bonner (16) for parsley cytochrome f and most c-type cytochromes (8, 35).

**Cytochrome C484.** This component was absorbed on the DEAE-cellulose column and was eluted with 50 mM NaCl, 10 mM phosphate (pH 7.5) and concentrated by rechromatography on DEAE-cellulose. This cytochrome was occasionally contaminated by residual phycocyanin but this pigment could be removed by a further salt fractionation.

**Ferredoxin-NADP" Reductase and Ferredoxin.** These proteins were eluted from the DEAE column by 0.1 M and 0.4 M NaCl respectively. They were not separated for the photoreduction experiments.

When the proteins were isolated from Swiss chard the procedure of San Pietro and Lang (28) was employed through the 75% acetone precipitation step. After dialysis, the proteins were further purified by the first DEAE-cellulose column treatment of Hill and Bendall's method (15).

**Results**

Reactions of photosynthetic electron transport and associated photophosphorylation were measured in *Phormidium luridum*. Cell-free preparations of the alga were prepared by either protoplast lysis or, alternatively, particles were isolated after grinding the cells with sand in solutions containing high concentrations of sucrose or ethylene glycol.

**Photoreactions of Lysed Protoplasts.** In an attempt to retain as much enzymic activity as possible, metabolically active algal protoplasts were prepared and added to the reaction mixtures of interest at the start of the reaction periods. The ensuing 8-fold decrease in stabilizing osmoticum caused the protoplasts to rupture thus instantaneously creating a cell-free preparation in the reaction vessel at the start of the reaction period. This technique eliminated the prolonged exposure of membranes to buffers and
consequent loss of activity during the work-up of homogenates as prepared by conventional methods.

Table I illustrates the capability of the algal preparations to sustain high rates of photophosphorylation coupled to the photoreduction of NADP⁺ or ferricyanide. When NADP⁺ is electron acceptor, the P/2e⁻ is close to unity indicating a considerable preservation of activity in these preparations. This ratio is, however, most likely slightly overestimated because of the incomplete recovery of NADPH in these determinations. Hoch and Martin (17) and Sauer and Biggins (29) showed the back-reaction to be significantly high during photoreduction by chloroplasts. It is possible that some oxidation of the NADPH occurred during the experiments reported here and work-up of the reaction mixtures. Nevertheless, the P/2e⁻ ratio for photophosphorylation coupled to ferricyanide reduction is high and comparable with the better measurements made with higher plant chloroplasts.

The reaction rates for NADP reduction are quite high for the open system with water as ultimate electron donor. The inhibition by the herbicide DCMU, implicates the involvement of photosystem 2 and the recovery of activity in the presence of added electron donor is similar to that for higher plant chloroplasts and algae, the reconstituted Anabaena system (31) and Anacystis nidulans (5,11).

It is interesting to note that the inclusion of supplementary ferredoxin and ferredoxin: NADP⁺- reductase, prepared from either Phormidium or Swiss chard, does not lead to any improvement of the rate of NADP⁺ photoreduction or the associated photophosphorylation by these lysed protoplasts (table I). This suggests that the endogenous enzymes are saturating for the reaction conditions employed. For this reason we were hopeful of demonstrating a cyclic photophosphorylation mediated by such endogenous components. We were, however, unable to achieve a significant synthesis of ATP via a cyclic process, even in the presence of DCMU and under anaerobic conditions, unless a conventional cyclic-cofactor was added (table II). We would suggest, therefore, that the ferredoxin-mediated cyclic photophosphorylation described by Tagawa, Tsujimoto and Arnon (33) is operating under what we would consider to be abnormally high concentrations of ferredoxin.

The effectiveness of PMS and DPIP as media-

Table I. Non-cyclic Photophosphorylation by Lysed Protoplasts

<table>
<thead>
<tr>
<th>Additions</th>
<th>Photophosphorylation</th>
<th>Photoreduction</th>
<th>P/2e⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. NAD⁺</td>
<td>132</td>
<td>149</td>
<td>0.855</td>
</tr>
<tr>
<td>2. NAD⁺, DCMU</td>
<td>5</td>
<td>8</td>
<td>...</td>
</tr>
<tr>
<td>3. NAD⁺, DCMU, DPIP, ascorbate</td>
<td>121</td>
<td>163</td>
<td>...</td>
</tr>
<tr>
<td>4. NAD⁺, ferredoxin, reductase</td>
<td>125</td>
<td>156</td>
<td>0.8</td>
</tr>
<tr>
<td>5. Ferricyanide</td>
<td>181</td>
<td>193</td>
<td>0.94</td>
</tr>
<tr>
<td>6. Ferricyanide, DCMU</td>
<td>15</td>
<td>24</td>
<td>...</td>
</tr>
</tbody>
</table>

Table II. Cyclic Photophosphorylation by Lysed Protoplasts

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Photophosphorylation</th>
<th>μmoles ATP/mg Chl/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMS</td>
<td>N₂</td>
<td>201</td>
</tr>
<tr>
<td>PMS</td>
<td>DCMU</td>
<td>188</td>
</tr>
<tr>
<td>PMS</td>
<td>N₂</td>
<td>238</td>
</tr>
<tr>
<td>PMS</td>
<td>air</td>
<td>202</td>
</tr>
<tr>
<td>PMS</td>
<td>Ascorbate</td>
<td>357</td>
</tr>
<tr>
<td>PMS</td>
<td>Ascorbate, air</td>
<td>354</td>
</tr>
<tr>
<td>PMS</td>
<td>Ascorbate</td>
<td>431</td>
</tr>
<tr>
<td>PMS</td>
<td>Ascorbate, air</td>
<td>187</td>
</tr>
<tr>
<td>DPIP</td>
<td>N₂</td>
<td>10</td>
</tr>
<tr>
<td>DPIP</td>
<td>Ascorbate, air</td>
<td>1.3</td>
</tr>
<tr>
<td>No cofactor</td>
<td>DCMU</td>
<td>1.1</td>
</tr>
<tr>
<td>No cofactor</td>
<td>air</td>
<td>0.04</td>
</tr>
</tbody>
</table>
tors of cyclic photophosphorylation by lysed pro-
plasts is shown in table II. Ascorbate relieves the
inhibitory action of air in the PMS system, but air
is necessary for full activity when DPIP is cofactor.
The activity is not appreciably diminished by relatively
high concentrations of DCMU indicating that the
reaction is a true cyclic process proceeding in the
absence of photosystem 2. Thus, the blue-green algal
mechanism is essentially similar to that of higher
plant chloroplasts and green algae. We do, however,
confirms the observation of Duane, Hohl and Krog-
mann (7) that the pH response for cyclic photophos-
phorylations mediated by PMS is anomalous with a
major peak at pH 7.0 and a minor peak in the
vicinity of pH 8.0.

Photoreactions of Particles Obtained by Grinding.
In agreement with reports on blue-green algae by
previous investigators (7, 27), we found that particles
prepared from Phormidium luridum by conventional
methods of cell disruption in the presence of a high
concentration of sucrose or ethylene glycol were
active in cyclic photophosphorylation. High yields of
broken cells were obtained from Phormidium by
grinding a wet cell paste in a mortar with pestle and
sand, and lamellar fragments were isolated from the
homogenate by differential centrifugation. Table III
compares the activity of particles obtained by this
method as isolated in either buffered sucrose or
ethylene glycol as grinding medium. The light
particles obtained by high speed centrifugation were
much more active than the heavy particles, irrespec-
tive of the grinding buffer used. In general, it was found
that the ethylene glycol medium was more suitable
than sucrose and rates from 250 to 450 μmoles/mg
Chl/hr for PMS cyclic photophosphorylation were
typically observed.

The optimum concentration of PMS necessary for
cyclic photophosphorylation by the light particles was
found to be 0.1 mm rather than 33 μm which is
optimal for lysed algal protoplasts and higher plant
chloroplasts.

We were unable to demonstrate non-cyclic photophos-
phorylation coupled to the photoreduction of NADP+
or ferricyanide by these particles. Duane, Hohl and Krog-
mann were also unable to demonstrate non-cyclic photophosphorylation by similarly prepared
particles from Anabaena (7). It appears, therefore,
that non-cyclic photophosphorylation is much more
susceptible to physical disruption and can only be
demonstrated when the algae are broken by relatively
mild techniques.

Effect of Washing the Algal Particles. If the
light particles prepared in ethylene glycol medium are
washed in the grinding buffer, the majority of the
soluble fraction of the cell is removed. The
soluble fraction contains the accessory pigment,
phycocyanin, the soluble cytochromes type c (554,
Phormidium) and type c (549, Phormidium), ferre-
doxin and ferredoxin: NADP+ reductase. As shown
in table IV, the cyclic photophosphorylation activity
was unaltered by this treatment. However, a sub-
stantial loss in activity (90%) occurred when the

Table IV. Cyclic Photophosphorylation: Effect of Washing the Particles or Protoplasts; Reconstitution by a
Soluble Protein

Conditions were as in table II using PMS as a cofactor. Protoplasts or particles contained 25 μg chlorophyll
a. The protein added back was the 50 to 100% saturated ammonium sulfate precipitate from the soluble fraction
of the cell.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate of photophosphorylation (μmoles ATP/mg Chl/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Light particles:</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>291</td>
</tr>
<tr>
<td>Once washed, ethylene glycol buffer</td>
<td>289</td>
</tr>
<tr>
<td>Twice washed, ethylene glycol buffer</td>
<td>280</td>
</tr>
<tr>
<td>Once washed, 50 mm tris</td>
<td>20</td>
</tr>
<tr>
<td><strong>Protoplasts:</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>230</td>
</tr>
<tr>
<td>Once washed, 50 mm tris</td>
<td>21</td>
</tr>
<tr>
<td>Twice washed, 50 mm tris</td>
<td>9</td>
</tr>
<tr>
<td>Twice washed plus active component</td>
<td>94</td>
</tr>
</tbody>
</table>
particles were washed with dilute buffer. A similar loss in activity was also observed when the algal protoplasts were lysed in a 40-fold excess of dilute buffer. Furthermore, the membranes obtained from protoplasts lysed in this fashion simultaneously lost their capacity for non-cyclic photophosphorylation when either ferricyanide or NADP$^+$ was used as electron acceptor. The phosphorylation coupled to NADP$^+$ photoreduction was not restored by the addition of supplementary ferredoxin and ferredoxin: NADP$^+$ reductase.

Reconstitution and Fractionation of an Active Component. In the case of the PMS-mediated cyclic photophosphorylation it was possible to restore some 15% of the activity lost on aqueous washing by addition of the entire soluble fraction of the cell. The component of interest was partially purified by fractionation of an aqueous extract from 100 g wet weight of acetone-treated cells prepared as described in Methods.

The 50 to 100% saturated ammonium sulfate sediment was dialyzed and then subjected to bulk fractionation on DEAE cellulose. The protein was loaded on a column equilibrated with 100 mm phosphate (pH 7.5). After washing with 25 mm buffer to remove cytochrome C$_{554}$ and plastocyanin, the column was eluted with 0.3 m NaCl. 50 mm phosphate (pH 7.5) and the eluate retained and dialyzed against 10mm phosphate (pH 7.5). In addition to the active component of interest, this fraction contained cytochrome C$_{554}$, phytoflavin and ferredoxin.

The dialyzed protein was then re-chromatographed using DEAE-cellulose. A column, 2 × 25 cm equilibrated with 10 mm phosphate (pH 7.5) was charged with the protein and was eluted with a gradient of NaCl increasing linearly from 0 to 0.6 m, 10 mm phosphate (pH 7.5). The total volume of the gradient was 400 ml, and 1.5 ml fractions were collected. Figure 3 shows the distribution of protein in the eluted fractions and their activity in the restoration of cyclic photophosphorylation. The active fraction appears to be a single component and is eluted by about 0.2 m NaCl. Difference spectrophotometry did not reveal the presence of cytochrome C$_{559}$ which is present in the first peak chromatographing at about 0.1 m NaCl. Ferredoxin was eluted by 0.3 m NaCl and the protein which is eluted by 0.25 m NaCl is thought to be phytoflavin.

Figure 4 shows the dependence of cyclic photophosphorylation on the active component, and the restorative activity is compared with that of the known soluble redox intermediates of the algal photosynthetic apparatus in table V. It is apparent that both cytochrome C$_{554}$ and plastocyanin are inactive and they do not appear to improve the activity of the component when added simultaneously.

The active component is a protein and the purest fractions available so far represent a 150-fold increase in purity capable of restoring some 40 to 50% of the PMS cyclic photophosphorylation. Difference spectrophotometry confirms the absence of a haemoprotein, but we have observed the presence of some flavin. The flavin content was variable and it is possible that this could be contamination from the adjacent flavoprotein in the chromatogram which is thought to be phytoflavin.

Membrane-bound Components. From the data presented above we can conclude that the washed algal membranes require a soluble protein for activity in cyclic photophosphorylation. The protein does not appear to be a known intermediate of the photosynthetic electron transfer chain and the question arises
Table V. Cyclic Photophosphorylation. Reconstitution by the Active Component and Comparison with Known Redox Intermediates

Conditions were as in table II using PMS as cofactor. Protoplasts or once-washed (50 mM Tris) membranes contained 25 µg chlorophyll a. The active component was 30 µg protein purified by salt gradient chromatography as in figure 3.

<table>
<thead>
<tr>
<th>Components added</th>
<th>Rate of photophosphorylation µmoles ATP/mg Chl/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoplasts</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Washed membranes</td>
<td>Active component</td>
</tr>
<tr>
<td>Washed membranes</td>
<td>Cytochrome c₅₅₄</td>
</tr>
<tr>
<td>Washed membranes</td>
<td>Cytochrome c₅₅₄ + plastocyanin</td>
</tr>
<tr>
<td>Washed membranes</td>
<td>Active component + cytochrome c₅₅₄</td>
</tr>
<tr>
<td>Washed membranes</td>
<td>Active component + plastocyanin + cytochrome c₅₅₄</td>
</tr>
</tbody>
</table>

Whether photophosphorylation by these washed membranes is truly proceeding in the complete absence of components such as cytochrome C₅₅₄, plastocyanin, and ferredoxin. These proteins are assumed to be removed from the membranes by the washing treatments.

Estimates based upon the recovery of cytochrome in the washings indicate that the aqueous treatment does lead to a complete removal. However, such measurements are probably erroneous because of the uncertainty of measuring low concentrations of haemoproteins in the presence of high concentrations of phycocyanin. Furthermore, the cytochrome chlorophyll ratio was found to vary considerably depending upon the age of the algal culture. We, therefore, analyzed the washed membranes directly by low temperature difference spectrophotometry. The membranes were extracted with 80% acetone at –20°C to remove interferring pigments such as chlorophyll. They were finally resuspended in sucrose to enhance the intensity of the absorption bands at liquid nitrogen temperature (35).

Representative difference spectra of the membranes obtained at temperatures close to that of liquid nitrogen are shown in figure 5. Spectrum C is a reduced minus untreated difference spectrum measuring all components which are oxidized in the untreated condition i.e., as extracted. This spectrum is that of cytochrome b₅. The small shoulder at 562 mm is suggestive of another component and the asymmetry to the blue of the main α band is thought to be a contribution by a small fraction of cytochrome C₅₅₄ which is oxidized in the untreated condition.

All components in the reduced form as extracted are shown in figure 5B, which is an untreated minus oxidized difference spectrum. The spectrum is of a bound form of cytochrome C₅₅₄ with a well-defined α₂ band at 548 mm due to splitting of the α band at low temperature. The possibility that this 548 mm band is the α band of cytochrome C₅₄₀, and not the α₂ band of cytochrome C₅₅₄, is ruled out on the grounds that cytochrome C₅₄₀ is very autooxidizable (18, 19, 20) and, therefore, would not appear in an untreated minus oxidized difference spectrum. Unless the acetone treatment selectively destroyed a membrane-bound form, we can also conclude that cytochrome C₅₄₀ is a totally soluble protein.

The relative intensities for the α₁ and α₂ bands of the soluble cytochrome C₅₅₄ (fig 2) and the membrane bound form (fig 5B) are different. The α₂ band of the membrane bound form is considerably more intense than that of the soluble form and this may be a direct reflection of the differing physical environments of the 2 forms.

Figure 5A is a difference spectrum, reduced minus oxidized, showing all cytochromes present. Both cytochrome b₅ and cytochrome b₅₄ can be seen. A similar difference spectrum at room temperature does not resolve cytochrome C₅₅₄ as is also the case for spinach chloroplast lamellae (4).

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Fig. 5. Low temperature (liquid nitrogen) difference spectra of well-washed Phormidium membranes extracted with –20°C, 80% acetone. Samples were oxidized with ferricyanide and reduced with dithionite. The solvent was 0.4 M sucrose. A) Reduced minus oxidized; B) untreated minus oxidized and C) reduced minus untreated.
Repeated washing of the membranes with dilute buffer, sonication or treatment with 1% digitonin for 1 hour did not alter the $\alpha$ band absorbancy ratio $b_\alpha/C_{554}$ which was generally 3.5 to 4. This implies that although a considerable fraction of the complement of cytochrome $C_{554}$ is soluble, a constant amount relative to cytochrome $b_\alpha$ is a lamellar component and is firmly bound to the membrane. The soluble form of this cytochrome constitutes in the order of 75% of the total depending upon growth conditions and it is not necessary for cyclic photophosphorylation. We would suggest that the membrane-bound form is probably of functional significance in the photoreactions.

**Discussion**

Our data indicate that the osmotic lysis of protoplasts is an excellent method for the creation of cell-free preparations from the blue-green alga, _Phormidium luridum_. The lysed protoplasts are active in cyclic photophosphorylation and photophosphorylation coupled to the Hill reaction and the photoreduction of NADP$^+$. Previous investigators have reported that preparations of certain blue-green algae are incapable of non-cyclic photophosphorylation. Duane, Hohl, and Kroghmann reported that extracts of _Anabaena variabilis_ were inactive in non-cyclic photophosphorylation (7). The same preparations were, however, very active in the Hill reaction (31), NADP$^+$ photoreduction (32) and cyclic photophosphorylation (7). Similarly, Black, Fewson, and Gibbs (5) were unable to demonstrate a coupling of phosphorylation to NADP$^+$ reduction by _Anacystis_ preparations (5).

More recently, Gerhardt and Trebst have prepared cell-free preparations from lyophylized _Anacystis nidulans_ (11). Aqueous extracts of these cells were found to be active in non-cyclic photophosphorylation coupled to ferricyanide reduction and NADP$^+$ reduction. The rates and coupling ratios observed were low, however.

It would appear, therefore, that the enzymatic apparatus for non-cyclic photophosphorylation in blue-green algae is very susceptible to uncoupling. The high rates we obtain for this activity by the _Phormidium_ system are a measure of the suitability of protoplast lysis for the preparation of algal extracts. The P/2e$^-$ ratios we observe for non-cyclic photophosphorylation are comparable to those the majority of investigators obtain using chloroplasts from higher plants.

Lysed protoplasts of _Phormidium_ are unusual in that supplementary ferredoxin and the associated flavoprotein necessary for NADP$^+$ photoreduction do not improve electron flow. This would suggest that the endogenous enzymes are not detached from their functional sites on the photosynthetic lamellae during the reaction period under the conditions we have employed. This is a further indication that at least a temporary preservation of intracellular integrity is maintained upon lysis of the algal protoplasts. When the protoplasts are disrupted in a large volume of dilute buffer and gently homogenized, however, these enzymes are removed from the lamellae and can be purified from the washings.

At this juncture, we are uncertain of the morphology of the chlorophyll-bearing cytoplasmic lamellae released upon lysis of the algal protoplasts. In the preparative centrifuge all the lamellae are sedimented in very low gravitational fields, suggesting that they are large membranes. Osmotic lysis may, indeed, liberate the complete unfragmented cytoplasmic lamellar system, and it is possible that the only membrane to undergo extensive damage is the constraining cell envelope.

The mechanism of cyclic photophosphorylation in _Phormidium luridum_ appears to be similar to that in _Anabaena variabilis_ investigated by Petrack and Lippmann (27) and described in detail by Duane, et al. (7). The response to cofactors, inhibitors and gas phase is the same and comparable to the higher plant mechanism. The apparatus for cyclic photophosphorylation is much more resistant to physical disruption than is the apparatus for non-cyclic photophosphorylation in blue-green algae.

The PMS and DPIP mediated photophosphorylation is catalyzed by algal particles prepared by conventional methods of cell disruption such as grinding and blending. In agreement with previous investigators (7,10), we observe that the cyclic photophosphorylation activity is not impaired by extensive washing of the particles. The washing removes the bulk of the soluble fraction of the cell which includes the accessory pigment, phycocyanin, the soluble cytochromes type $C_{540}$ and $C_{554}$, and ferredoxin. Susor and Kroghmann (31) and Gerhardt and Santo (10) have shown that phycocyanin is also not essential for the Hill reaction. It is, therefore, possible that this pigment is not an integral component of quantum conversion. It is most likely functional solely in the harvesting of radiation, the energy of which is then transferred to chlorophyll.

Although cyclic photophosphorylation activity is resistant to extensive washing with solutions containing sucrose or ethylene glycol (7,10) we observe that it is diminished when the membranes are washed with dilute buffers. The loss in activity is due to the removal of a soluble protein which, when added back to the washed membranes, quantitatively restores the photophosphorylation. This component is not removed from the membrane by washing with sucrose or ethylene glycol solutions. It could be an intermediate of the electron transfer chain or a factor necessary for the coupling of ATP synthesis to electron flow.

The protein has been partially purified and does not appear to be a known redox intermediate such as plastocyanin, ferredoxin, cytochrome $C_{554}$, or cytochrome $C_{540}$. Its possible functional equivalence to the _Anacystis_ factor (9) will be resolved by our
current studies on the complete purification and characterization of the component.

Our spectrophotometric studies of the well-washed membranes show the presence of cytochrome \( b_6 \) and a bound form of the high potential cytochrome \( C_{554} \). The difference spectra are virtually identical to those obtained from the lamellae of higher plant chloroplasts (4, 16). The \( \alpha \) band absorbancy ratio \( b_6/C_{554} \) and the peak maxima are the same. We were unable to remove the bound form of cytochrome \( C_{554} \) from the algal particles by sonication or treatment with digitonin. Wessels has recently reported that spinach cytochrome \( f \) is removed from chloroplast lamellae by digitonin treatment (34). This may represent an important difference in the molecular architecture of chloroplast lamellae and algal membranes.

The bound form of cytochrome \( C_{554} \) is spectrophotometrically different from the soluble form at liquid nitrogen temperature. The \( \alpha \) band of the bound form is more intense than that of the soluble form and this may be due to the difference in physical environment. The splitting of the \( \alpha \) band of cytochrome \( C_{554} \) at low temperature is typical of most C-type cytochromes including those from mammalian sources (8, 35) and photosynthetic tissues of higher plants and algae. Hill and Bonner (16) showed that the \( \alpha \) band of purified parsley cytochrome \( f \) and membrane bound spinach cytochrome \( f \) split at liquid nitrogen temperature. We have shown (unpublished observations) that the \( \alpha \) band of cytochrome type \( c \) (552, Euglena) is also resolved into \( \alpha_1 \) and \( \alpha_2 \) bands at low temperature. The cytochrome \( C_2 \) from \( \text{Rhodospirillum rubrum} \) does not behave in this fashion (8).

Thus, the cytochrome \( C_{554} \) from \( \text{Phormidium luridum} \) resembles the photosynthetic cytochromes of type \( c \) from eucaryotes rather than those from procaryotic cells.

We do not have a precise chlorophyll:cytochrome ratio for the \( \text{Phormidium} \) membranes because of the uncertainty of the extinction coefficients of the cytochromes at liquid nitrogen temperature. This uncertainty is a combination of the contribution of band sharpening, solvent enhancement, and the slight increase in optical path length due to freezing (8, 35). From the room temperature difference spectra we obtained, which are poor in that cytochrome \( C_{554} \) is not resolved, we estimate that the cytochrome \( b_6 \)-chlorophyll ratio is 1:500.

It is highly probable that the membrane-bound form of cytochrome \( C_{554} \) in \( \text{Phormidium} \) is of functional significance in the photosynthetic mechanism and is the equivalent of cytochrome \( f \) which is present in higher plant chloroplast lamellae. The physiological function of the soluble form of cytochrome \( C_{554} \) is less predictable. Our studies indicate that it is not necessary for cyclic photophosphorylation and, therefore, the operation of photosystem I.

Holton and Myers found that the relative concentrations of soluble cytochrome \( C_{554} \) and chlorophyll \( a \) was constant and independent of growth conditions in \( \text{Anacystis nidulans} \) (18, 20). In contrast to this finding, we observed that the bound-cytochrome:chlorophyll \( a \) ratio was constant and the soluble cytochrome:chlorophyll \( a \) ratio was variable. In rapidly growing exponential phase cells, the soluble cytochrome \( C_{554} \) concentration was relatively low compared to stationary phase cells. In this respect, \( \text{Phormidium luridum} \) is somewhat similar to \( \text{Chlamydomonas reinhardi} \) (13).

In that the blue-green algae have a soluble form of cytochrome \( C_{554} \), they are similar to other algal divisions. \( \text{Porphyra tenera} \) (21, 22), \( \text{Euglena gracilis} \) (26) and \( \text{Chlamydomonas reinhardi} \) (13) have readily extractable high potential cytochromes of type \( c \) which are associated with the photosynthetic apparatus. Gorman and Levine (14) report that some fraction of the \( \text{Chlamydomonas} \) cytochrome \( C_{554} \) is membrane bound, and, at room temperature, is spectrophotometrically different from the soluble form. It would be of interest to know if \( \text{Euglena} \) and \( \text{Porphyra} \) also have a membrane-bound cytochrome \( c \) associated with the photosynthetic lamellae. The available difference spectrum (reduced minus oxidized) of \( \text{Euglena} \) membranes does not reveal a bound form of cytochrome type \( c \) (552, \( \text{Euglena} \)) but only cytochrome \( b_{601} \) and cytochrome \( a_{903} \) (26). As is the case for spinach chloroplasl lamellae (4) and \( \text{Phormidium luridum} \) membranes, it is possible that the cytochrome of type \( c \) is not resolved in such a room temperature difference spectrum. In our experience, this cytochrome is only resolved in room temperature difference spectra of the type ferri-cyanide-oxidized minus untreated, or oxidized minus ascorbate-reduced. In both of these treatments, cytochrome \( b_6 \), which is usually in the oxidized form on extraction, is not seen and therefore facilitates the resolution of the cytochrome \( c \).

Under the conditions employed we have not detected a membrane-bound form of the low potential cytochrome type \( c \) (549, \( \text{Phormidium} \)) first discovered in \( \text{Anacystis nidulans} \) by Holton and Myers (18, 19, 20). The physiological function of this soluble cytochrome and also the soluble form of cytochrome \( C_{554} \) awaits further investigation.

In conclusion, our studies show that the mechanism of photosynthesis in blue-green algae is fundamentally similar to that of higher plants. Certain components related to the photosynthetic apparatus are soluble and readily removed from the chlorophyll-bearing lamellae. However, with the exception of the phosphorylation factor, they do not appear to be intrinsic or mandatory components in the structure or function of quantum conversion and reactions of electron transfer.

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Literature Cited


