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Photosynthetic Electron Transport in a Cell-Free Preparation from the Thermophilic Blue–Green Alga *Phormidium laminosum*

Alison C. STEWART and Derek S. BENDALL
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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1. A cell-free preparation of membrane fragments was prepared from the thermophilic blue–green alga *Phormidium laminosum* by lysozyme treatment of the cells followed by osmotic shock to lyse the spheroplasts. The membrane fragments showed high rates of photosynthetic electron transport and O_2 evolution (180–250 μmol of O_2/h per mg of chlorophyll *a* with 2,6-dimethyl-1,4-benzoquinone as electron acceptor). O_2-evolution activity was stable provided that cations (e.g. 10mM-Mg^{2+} or 100mM-Na^+) or glycerol (25%, v/v) were present in the suspending medium. 2. The components of the electron-transport chain in *P. laminosum* were similar to those of other blue–green algae: the cells contained Pigment P700, plastocyanin, soluble high-potential cytochrome *c*-553, soluble low-potential cytochrome *c*-549 and membrane-bound cytochromes *f*, *b*-563 and *b*-559 (both low- and high-potential forms). The amounts and midpoint potentials of the membrane-bound cytochromes were similar to those in higher-plant chloroplasts. 3. Although O_2 evolution in *P. laminosum* spheroplasts was resistant to high temperatures, thermal stability was not retained in the cell-free preparation. However, in contrast with higher plants, O_2 evolution in *P. laminosum* membrane fragments was remarkably resistant to the non-ionic detergent Triton X-100.

The blue–green algae (and one recently discovered strain of green alga; Withers *et al.*, 1977) are the only photosynthetic prokaryotes capable of oxygenic photosynthesis, i.e. the photo-oxidation of water by Photosystem 2 to produce molecular O_2. Studies on membrane preparations from several strains of blue–green algae have shown that in many respects the components and organization of the photosynthetic electron-transport chain are the same as in higher-plant chloroplasts (Krogmann, 1977), indicating that the blue–green-algal system is a good model for the study of oxygenic photosynthesis in general. In addition, some strains of blue–green algae can grow and photosynthesize at temperatures that completely inactivate O_2 evolution in higher plants, suggesting that such organisms might yield more stable O_2-evolving preparations than those from higher-plant chloroplasts. This is of major importance, since the chemical investigation of the O_2-evolving system, one of the main unsolved problems in the study of photosynthesis, has been hampered in higher plants by the extreme instability of the O_2-evolving complex not only to mild heating but to most of the techniques, particularly detergent treatment, that are used for the chemical fractionation of biological membranes.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid: Mes, 4-morpholine-ethanesulphonic acid.

There are several reports in the literature of photosynthetically active cell-free preparations from mesophilic blue–green algae (Susor & Krogmann, 1964; Biggins, 1967*a*; Arnon *et al.*, 1974; Binder *et al.*, 1976) and from the 'semi-thermophilic' *Anacystis nidulans*, which will grow at temperatures up to about 45°C (Fredericks & Jagendorf, 1964; Ono & Murata, 1978), but, apart from one brief report (Yamaoka *et al.*, 1978), no active well-characterized cell-free preparations from truly thermophilic blue–green algae have been described; such a preparation is a necessary first step towards the eventual isolation of an active O_2-evolving particle from this group of organisms.

The present paper describes the preparation and properties of photosynthetically active membrane fragments from the thermophilic blue–green alga *Phormidium laminosum*, a lysozyme-sensitive filamentous strain that grows in hot springs at temperatures up to 57–60°C (Castenholz, 1970). The preparation has been characterized with respect to the components of its electron-transport chain, rates of electron transport and the stability of O_2

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evolution towards high temperatures and detergent treatment.

A preliminary report of this work was presented at the 4th International Congress on Photosynthesis (Stewart & Bendall, 1977).

Materials and Methods

Algal strains and growth conditions

Phormidium laminosum (strain OH-1-p.Cl 1), obtained from Dr. R. W. Castenholz (Department of Biology, University of Oregon, Eugene, OR, U.S.A.), was grown on Medium D of Castenholz (1970), supplemented with $NaHCO_3$ (0.5g/l). Cultures were grown at 45°C in a Gallenkamp Orbital Incubator at a shaking speed of 110 cycles/min, with continuous illumination from four 30W fluorescent tubes, and in an atmosphere enriched with 5% CO_2.

'*Anacystis nidulans*' (Culture Centre of Algae and Protozoa, Storey's Way, Cambridge, U.K.; strain 1405/1, recently reclassified as a *Synechococcus* strain) was grown at 37°C on Medium BG-11 of Stanier *et al.* (1971).

Preparation of spheroplasts and membrane fragments from Phormidium laminosum

The method used was similar to that described by Binder *et al.* (1976) for the mesophilic blue–green alga *Phormidium luridum*. Five litres of culture in late exponential phase (5–6 days' growth) were harvested by centrifugation, washed once and resuspended in 100ml of 'buffer A', containing 0.5M-sorbitol, 10mM-$MgCl_2$, 10mM-Hepes/NaOH, pH 7.5, and 5mM-phosphate buffer (NaH_2PO_4/K_2HPO_4), pH 7.5, plus 12.5mM-EDTA (disodium salt). Solid lysozyme (BDH Chemicals, Poole, Dorset, U.K.) was added, to a concentration of 0.1% (w/v), and the mixture was homogenized in a Polytron blender (Kinematica G.m.b.H., Luzern, Switzerland) for 2–3s at setting 3. This had the effect of breaking up large clumps of tangled filaments, and appeared to enhance access of lysozyme to the cells. The mixture was incubated for 1h at 37°C in room light in a shaking water bath (shaking speed 120 cycles/min).

After this time, microscopic examination showed the appearance of single spheroplasts and sometimes chains of spheroplasts up to about five cells in length. The lysozyme reaction was stopped by diluting the preparation with 300ml of ice-cold buffer A (without EDTA) and centrifuging at 3000*g* for 7min. The supernatant, which was pale blue, was discarded.

For the preparation of spheroplasts, the pellet was gently resuspended in a small volume of buffer A, and centrifuged at 200*g* for 90s. The pellet, consisting of filaments unaffected by lysozyme, was discarded. The supernatant was centrifuged again at 3000*g* for 7min, and the pellet of spheroplasts was gently resuspended in the minimum volume of buffer A. Spheroplasts remained intact (as judged under the light-microscope) for about 2h when stored at 4°C.

For the preparation of membrane fragments, the pellet obtained at the end of lysozyme treatment was resuspended in 400ml of ice-cold hypo-osmotic 'buffer B', containing 10mM-$MgCl_2$, 10mM-Hepes/NaOH buffer, pH 7.5, and 5mM-phosphate buffer (NaH_2PO_4/K_2HPO_4), pH 7.5, and thoroughly homogenized in a glass homogenizer. The mixture was centrifuged at 200*g* for 90s (to pellet unaffected filaments) and the supernatant was filtered through a sintered-glass filter (no. 1 porosity). The filtrate was centrifuged at 27000*g* for 10min and the deep-blue supernatant, containing the bulk of the accessory phycobilin pigments, was discarded. The pellet of membrane fragments was washed once and resuspended in the minimum volume of buffer A. The final yield of membrane fragments, expressed as recovery of chlorophyll *a*, was 70–90%.

Membrane fragments remained active in electron transport for over 6h when stored at 4°C, and could be stored for several months in liquid N_2 without loss of activity.

Membrane fragments from Anacystis nidulans

Two litres of culture were harvested and washed, and the cells were resuspended in 20–40ml of a buffer containing 0.5M-mannitol, 30mM-potassium phosphate buffer, pH 6.8, and 1mM-EDTA. The cell suspension was treated with 0.1% (w/v) lysozyme for 2h at 35°C, then centrifuged, washed and resuspended in 30ml of ice-cold 'buffer C', containing 0.4M-mannitol, 10mM-$MgCl_2$, 5mM-NaCl and 30mM-potassium phosphate buffer, pH 7.5. The cell suspension was passed twice through a pre-cooled French pressure cell at a pressure of 3–4MPa (300–400kg/cm^2) and the extruded mixture was centrifuged at 3000*g* for 10min to pellet unbroken cells. Membrane fragments were collected from the supernatant by centrifuging at 38000*g* for 10min, and resuspended in the minimum volume of buffer C.

Preparation of pea chloroplasts

Peas (*Pisum sativum*, var. Feltham First) were grown in moist vermiculite in a greenhouse. Chloroplasts were isolated from the 2–3-week-old seedlings by the method of Cockburn *et al.* (1968).

Chlorophyll determination

In blue–green-algal preparations, chlorophyll *a* concentration was measured by the method of Arnon *et al.* (1974).

For pea chloroplasts, total chlorophyll ($a+b$) concentration was measured by the method of Arnon (1949).

Measurement of electron-transport activities

Rates of O_2 evolution or uptake were measured with a Hansatech oxygen electrode (Hansatech, King's Lynn, Norfolk, U.K.) calibrated with air-saturated distilled water at 25°C (the optimum temperature for O_2 evolution in *P. laminosum* and *Anacystis nidulans* membrane fragments) or 20°C (for pea chloroplasts). The reaction mixture contained, in a final volume of 1.0ml of buffer A (pH 7.5), chloroplasts, spheroplasts or membrane fragments equivalent to 10μg of chlorophyll. The chamber was illuminated with saturating red light (intensity 90mW/cm^2) from a 150W tungsten/halogen projector lamp filtered through a Schott RG610 filter.

Photosystem 2 activity was measured as O_2 evolution with 1mM-2,6-dimethyl-1,4-benzoquinone (Aldrich Chemicals, Milwaukee, WI, U.S.A.) as electron acceptor, kept oxidized by 2mM-$K_3Fe(CN)_6$.

Photosystem 1 activity was measured as O_2 uptake with 75μM-2,6-dichlorophenol-indophenol, 2.5mM-sodium D-isoascorbate, 10μM-3-(3,4-dichlorophenyl)-1,1-dimethylurea, 3mM-NH_4Cl, 30μg of Methyl Viologen/ml and 2μl of catalase suspension (BDH Chemicals).

Electron-transport activity through both photosystems was measured as O_2 uptake with 30μg of Methyl Viologen/ml and 1mM-NaN_3, or as O_2 evolution with 2mM-$K_3Fe(CN)_6$. Uncoupled rates for these reactions were obtained by adding 3.3mM-NH_4Cl.

Stock solutions of 100mM-2,6-dimethyl-1,4-benzoquinone and 1mM-3-(3,4-dichlorophenyl)-1,1-dimethyl urea were made up in AnalaR ethanol.

Assay of membrane-bound cytochromes

Membrane-bound cytochromes were assayed by the method of Bendall *et al.* (1971). Reduced-minus-oxidized difference spectra were recorded at room temperature or at 77K with a sensitive split-beam spectrophotometer (Johnson Foundation, University of Pennsylvania, Philadelphia, PA, U.S.A.), with membrane fragments at a concentration of 80–90μg of chlorophyll a/ml. For recording difference spectra of cytochrome f it was found that 2% Triton X-100, rather than 1% as recommended by Bendall *et al.* (1971) for higher plants, was needed to destroy all the high-potential cytochrome b-559 in *P. laminosum*. A molar absorption difference coefficient (reduced minus oxidized) of $\Delta\varepsilon$ 20000 M$^{-1}\cdot$cm^{-1} was assumed for all the cytochromes.

In one experiment, menadiol–1,4-benzoquinol and dithionite–menadiol difference spectra were recorded. The following procedure was used. Membrane fragments at a concentration of 100μg of chlorophyll a/ml in 0.5M-sorbitol/10mM-$MgCl_2$/100mM-Mes/NaOH buffer, pH 6.0, were kept anaerobic by a flow of O_2-free N_2, and then the suspension was divided equally between two cuvettes. 1,4-Benzoquinol (1mM) was added to both cuvettes, and a base-line was obtained before the difference spectra were recorded. Menadiol (a gift from Dr. P. Rich) was added from a freshly prepared solution to a final concentration of 1mM, and $Na_2S_2O_4$ was added as a few grains of the solid. It has been estimated that under these conditions menadiol reduces at least 80% of low-potential cytochrome b-559, but only about 3% of cytochrome b-563, so that menadiol–1,4-benzoquinol and dithionite–menadiol difference spectra allow the two low-potential cytochromes to be measured separately (Rich & Bendall, 1980).

Redox potentiometry

Membrane fragments at a concentration of 100μg of chlorophyll a/ml, in 0.5M-sorbitol/10mM-$MgCl_2$/100mM-Mes/NaOH buffer, pH 6.5, were kept anaerobic by a flow of O_2-free N_2, and the potential was monitored with a platinum electrode against a saturated-KCl/calomel reference electrode. The following redox mediators were added to the sample cuvette: 20μM-1,4-benzoquinone, 20μM-2-methyl-1,4-benzoquionone, 20μM-2,3-dimethyl-1,4-benzoquinone, 25μM-duroquinone, 25μM-diaminodurene, 12.5μM-phenazine methosulphate, 12.5μM-phenazine ethosulphate, 25μM-2-hydroxy-1,4-naphthoquinone, 10μM-5-hydroxy-1,4-naphthoquinone, 20μM-2-methyl-1,4-naphthoquinone, 5μM-anthroquinone and 20μM-anthroquinone-2,6-disulphonate. Catalase [approx. 400 units (μmol/min)/ml] was also included. The potential was altered by addition of small amounts of $Na_2S_2O_4$ (reductive) or $K_3Fe(CN)_6$ (oxidative).

The reference cuvette contained an identical sample of membrane fragments that had been reduced with 1mM-1,4-benzoquinol. The redox states of the cytochromes in the sample cuvette, relative to the reference cuvette, were monitored by scanning their spectra with the Johnson Foundation split-beam spectrophotometer.

Extraction of plastocyanin and cytochrome c-553

The method used was based on that of Wood (1977) for extraction of soluble cytochromes from green algae. Five litres of culture were treated with lysozyme as described above, but at the end of the incubation with lysozyme the washed spheroplasts were resuspended in 50ml of a 'low-salt' hypo-

osmotic medium containing 3mM-$MgCl_2$/10mM-potassium phosphate buffer, pH 7.0. The mixture was frozen for 1h at −80°C (in an ethanol/solid CO_2 bath), then thawed at 25°C and centrifuged at 38000*g* for 10min. The deep-blue supernatant was decanted and kept. The pellet was resuspended in 50ml of a 'high-salt' buffer (0.4M-NaCl/10mM-potassium phosphate buffer, pH 7.0), then frozen, thawed and centrifuged several times as described above. The supernatants, containing plastocyanin and soluble cytochrome as well as large amounts of phycocyanin, were pooled and dialysed overnight against 10mM-potassium phosphate buffer, pH 7.0.

The dialysed preparation was passed through a column (2.5cm × 10cm) of DEAE-cellulose (DE-52; Whatman Biochemicals, Maidstone, Kent, U.K.) equilibrated with 10mM-potassium phosphate buffer, pH 7.0. The acidic phycocyanin and low-potential cytochrome *c*-549 bound to this column, but the basic plastocyanin and cytochrome *c*-553 were eluted by the equilibration buffer. The eluate was concentrated to about 10ml by treatment with Aquacide (Calbiochem, Richmond, CA, U.S.A.).

Spectra were recorded on the split-beam spectrophotometer.

Pigment P-700

Pigment P-700 (the reaction centre of Photosystem 1) was assayed chemically by recording difference spectra [2mM-sodium D-isoascorbate − 1mM-$K_3Fe(CN)_6$], at room temperature, of membrane fragments at a concentration of 20μg of chlorophyll *a*/ml. An absorption difference coefficient of $\Delta\varepsilon_{727-700}$ $6.4 \times 10^3 M^{-1} \cdot cm^{-1}$ was assumed (Wood & Bendall, 1975).

Results

Electron-transport activities of Phormidium laminosum membrane fragments

Lysozyme digestion of *P. laminosum* cells produced spheroplasts that were active in electron transport through both photosystems (Table 1). When spheroplasts were broken hypo-osmotically in buffer B and membrane fragments were collected by centrifugation, the fragments retained high rates of O_2 evolution with 2,6-dimethyl-1,4-benzoquinone or $K_3Fe(CN)_6$ as electron acceptor, even though the bulk of the accessory pigment phycocyanin was removed when the spheroplasts were broken.

The rate of electron transport from water to Methyl Viologen was lower in fragments than in spheroplasts, suggesting that the fragments have lost a soluble component or components of the electron-transport chain between the photosystems. However, adding back equivalents of the supernatant did not restore the rate to that of the spheroplasts even if excess of supernatant was added together with brief sonication, which might allow components originally from the internal thylakoid space to return to their original locations. The fact

Table 1. *Electron-transport activities of spheroplasts and membrane fragments of Phormidium laminosum*

Electron-transport activities were measured at 25°C in the Hansatech oxygen electrode in a final volume of 1.0ml of buffer A, pH 7.5, containing spheroplasts or membrane fragments equivalent to 10μg of chlorophyll *a*. Final concentrations of reagents used were: 2mM-$K_3Fe(CN)_6$, 1mM-2,6-dimethyl-1,4-benzoquinone (DMBQ), 3.3mM-NH_4Cl, 30μg of Methyl Viologen (MeV)/ml, 75μM-2,6-dichlorophenol-indophenol (DCIP), 10mM-ascorbate (in ascorbate→O_2 system) or 2.5mM-ascorbate (with 2,6-dichlorophenol-indophenol), and 10μM-3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Other additions were: 2μl of catalase suspension to the 2,6-dichlorophenol-indophenol (reduced)→Methyl Viologen system, and 1mM-NaN_3 to the ascorbate→O_2, 2,6-dichlorophenol-indophenol (reduced)→O_2 and endogenous systems. Values listed are ranges over at least five experiments.

Reaction system	Rate of O_2 evolution or uptake* (μmol of O_2/h per mg of chlorophyll *a*)	
	Spheroplasts	Membrane fragments
$H_2O \rightarrow DMBQ$	220–270	180–250
$H_2O \rightarrow Fe(CN)_6^{3-}$	100–140	100–180
$H_2O \rightarrow Fe(CN)_6^{3-}$ (+NH_4Cl)	140–180	110–180
$H_2O \rightarrow MeV$	50–70	40–70
$H_2O \rightarrow MeV$ (+NH_4Cl)	90–100	45–75
$DCIPH_2 \rightarrow MeV$ (+DCMU)	250–300	350–440
$DCIPH_2 \rightarrow O_2$ (+DCMU)	100–200	130–200
Ascorbate→O_2	40–100	30–60
Ascorbate→O_2 (+DCMU)	30–60	30–60
Endogenous O_2 uptake	30–70	30–70

* O_2 evolution was observed in reaction systems with $K_3Fe(CN)_6$ or 2,6-dimethyl-1,4-benzoquinone as electron acceptor. O_2 uptake was observed when Methyl Viologen or O_2 was the electron acceptor.

that washed membrane fragments remained highly active in the Photosystem 1 partial reaction 2,6-dichlorophenol-indophenol (reduced)→Methyl Viologen suggests that in *P. laminosum* low concentrations of reduced 2,6-dichlorophenol-indophenol can donate electrons directly to Pigment P-700, so that the reaction is largely independent of plastocyanin and soluble cytochrome. This interpretation was supported by the observation that treatment with $HgCl_2$, which inhibits the oxidation of plastocyanin (Kimimura & Katoh, 1972), had little effect on the 2,6-dichlorophenol-indophenol (reduced)→Methyl Viologen reaction results not shown).

Both spheroplasts and membrane fragments of *P. laminosum* appeared to contain the 'O_2-reducing system' that has been noted in other blue–green algae (Fujita & Myers, 1967; Honeycutt & Krogmann, 1972), so that a significant rate of O_2 uptake could be observed without the addition of an autoxidizable Photosystem 1 acceptor such as Methyl Viologen. O_2 uptake could also be measured in a completely endogenous system with no added electron donor or acceptor (Table 1).

Addition of the uncoupler NH_4Cl stimulated electron transport from water to Methyl Viologen in spheroplasts, but usually had little effect on membrane fragments; the latter appeared to be largely uncoupled in most preparations.

The reaction conditions listed in the legend to Table 1 are those that were found to be optimal for measurement of electron-transport activities in *P. laminosum*. At least 5mM-Mg^{2+} was required in the reaction medium for maximum rates of O_2 evolution, and the best rates were obtained at 25°C in an Mg^{2+}-containing medium osmotically stabilized by 0.5M-sorbitol. Phosphate was not essential, and O_2 evolution was not stimulated by Ca^{2+}, in contrast with results presented by Binder *et al.* (1976) and Brand (1979) for other blue–green algae.

Effect of washing on O_2 evolution in P. laminosum membrane fragments

Many previous reports have indicated that in blue–green algae O_2 evolution is inactivated if membrane preparations are washed in dilute aqueous buffers (Fredericks & Jagendorf, 1964; Tel-Or & Avron, 1974; Tel-Or & Stewart, 1975; McSwain *et al.*, 1976). It was confirmed that *P. laminosum* membrane fragments lose O_2-evolution capacity if they are washed and stored in 20mM-Hepes buffer alone (Table 2). The loss of activity appeared to be irreversible, since adding back equivalents of the supernatant failed to restore activity. However, activity was preserved if 10mM-$MgCl_2$ was included in the washing and resuspension medium. $MgCl_2$ could be replaced by 10mM-$CaCl_2$ or 100mM-NaCl, and 25% (v/v) glycerol was also effective as a stabilizer of activity. Thus by the choice of appropriate conditions the O_2-evolving apparatus of *P. laminosum* could readily be maintained in a stable and active state.

Table 2. *Effect of washing in different media on oxygen evolution in Phormidium laminosum membrane fragments*

Samples (0.5ml) of membrane fragments containing 0.9mg of chlorophyll *a* were diluted to 20ml with 20mM-Hepes/NaOH buffer, pH 7.5, plus the additions shown, and kept at 4°C for 30min. The mixtures were centrifuged at 27000*g* for 15min, and the pellets were resuspended in 1ml of the same media used for washing. Rates of O_2 evolution, with 1mM-2,6-dimethyl-1,4-benzoquinone plus 2mM-$K_3Fe(CN)_6$ as electron acceptor, were measured immediately.

Additions to 20mM-Hepes/NaOH, pH 7.5	Rate of O_2 evolution (μmol of O_2/h per mg of chlorophyll *a*)
None	35
10mM-$MgCl_2$	241
10mM-$CaCl_2$	213
100mM-NaCl	234
25% (v/v) glycerol	239
Control (untreated)	240

Components of the electron-transport chain

Membrane-bound cytochromes. Reduced-minus-oxidized difference spectra of *P. laminosum* membrane fragments at room temperature and 77K (Figs. 1 and 2) indicated the presence of cytochromes *f*, *b*-563 and *b*-559, and in addition a low-potential cytochrome with an α-band at 549nm was observed (Fig. 1*a*). Redox titrations of fresh preparations showed that a high-potential form of cytochrome *b*-559 was present, with a midpoint potential of approx. 330–350mV. As in higher-plant chloroplasts, fresh preparations also contained smaller amounts of a lower-potential form of cytochrome *b*-559 that was reducible by menadiol but not by 1,4-benzoquinol (Fig. 3*a*) and had a midpoint potential between 0 and +100mV. Cytochrome *b*-563, reducible by dithionite but not by menadiol (Fig. 3*b*), had a midpoint potential between 0 and −100mV. Unfortunately, more accurate determinations of the midpoint potentials of low-potential cytochrome *b*-559 and cytochrome *b*-563 were not possible because of the presence of variable amounts of converted high-potential cytochrome *b*-559, which, unlike the higher-plant cytochrome, was unstable under the conditions of the titration and gradually decayed to forms with lower potentials.

Cytochrome *f* from *P. laminosum* had a midpoint potential of approx. 330–350mV, titrating together with high-potential cytochrome *b*-559, but differed

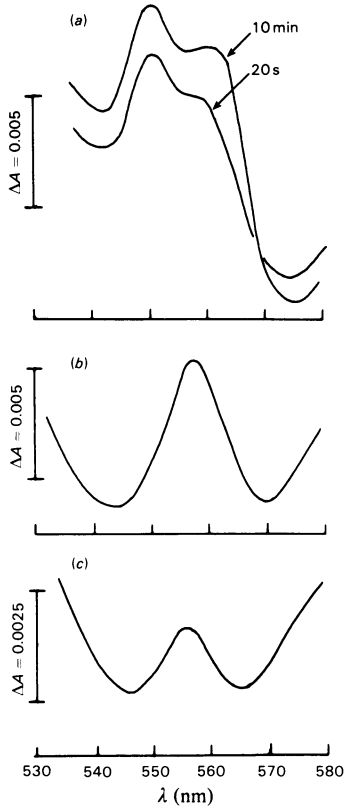

Fig. 1. *Room-temperature difference spectra of membrane-bound cytochromes in Phormidium laminosum* Membrane fragments were diluted with distilled water to a concentration of 226 μg of chlorophyll *a*/ml, then mixed with an equal volume of a buffer containing 0.6 M-mannitol, 2 mM-$MgCl_2$, 2 mM-$MnCl_2$ and 4 mM-EDTA in 100 mM-potassium phosphate buffer, pH 6.5, giving a final concentration of 113 μg of chlorophyll *a*/ml. Difference spectra (reduced-minus-oxidized) were recorded on the split-beam spectrophotometer as described by Bendall *et al.* (1971). The final concentrations of oxidants and reductants were: 1.25 mM-$K_3Fe(CN)_6$, 1.25 mM-1,4-benzoquinol, 0.5 mg of $Na_2S_2O_4$/ml (+400 units of catalase/ml). (*a*) $Na_2S_2O_4$ minus 1,4-benzoquinol. Spectra were recorded 20 s and 10 min after addition of $Na_2S_2O_4$ to the sample cuvette. (*b*) 1,4-Benzoquinol$-K_3Fe(CN)_6$ difference spectrum. (*c*) 1,4-Benzoquinol$-K_3Fe(CN)_6$ [+2% (w/v) Triton X-100] difference spectrum.

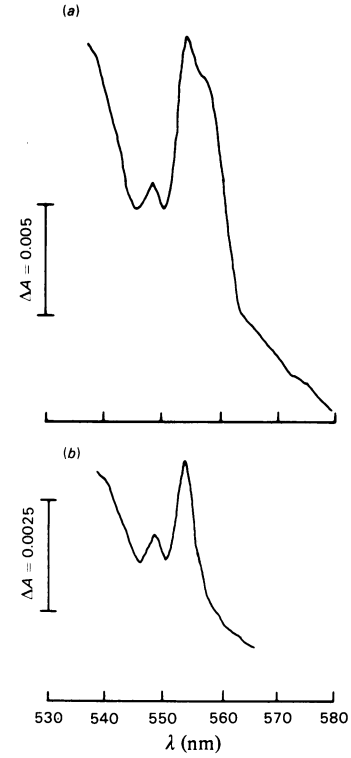

Fig. 2. *Low-temperature (77 K) difference spectra of membrane-bound cytochromes in Phormidium laminosum* Membrane fragments were prepared as described in Fig. 1, and spectra recorded at 77 K as described by Bendall *et al.* (1971). (*a*) 1,4-Benzoquinol$-K_3Fe(CN)_6$ difference spectrum. (*b*) 1,4-Benzoquinol$-K_3Fe(CN)_6$ [+2% (w/v) Triton X-100] difference spectrum.

from higher-plant cytochrome *f* in having its α-band at 555–556 nm rather than 554 nm (Fig. 1*c*) and in exhibiting a more pronounced splitting of the α-band at liquid-N_2 temperature (Fig. 2*b*).

Cytochrome *c*-549, with a very low midpoint potential of less than −250 mV, appeared to be similar to the low-potential soluble cytochrome observed by Biggins (1967*b*) in *P. luridum*. Washing in low-salt hypo-osmotic buffer or brief sonication failed to remove *P. laminosum* cytochrome *c*-549 from the membranes, but it was gradually removed by repeated freezing (at −80°C), thawing and centrifuging in a buffer containing 10 mM-phosphate, pH 7.0, plus 0.4 M-NaCl. Spectra of the membrane residue after this treatment (Fig. 4) still indicated the presence of bound cytochromes *f*, *b*-563 and *b*-559, although most of the cytochrome *b*-559 appeared to have been converted into a low-potential form by the freezing and thawing treatment.

The amounts and approximate midpoint potentials of the membrane-bound photosynthetic cytochromes in *P. laminosum* are summarized in Table 3.

Plastocyanin and cytochrome c-553. Plastocyanin, which was a basic protein in *P. laminosum*, could generally be extracted from spheroplasts by

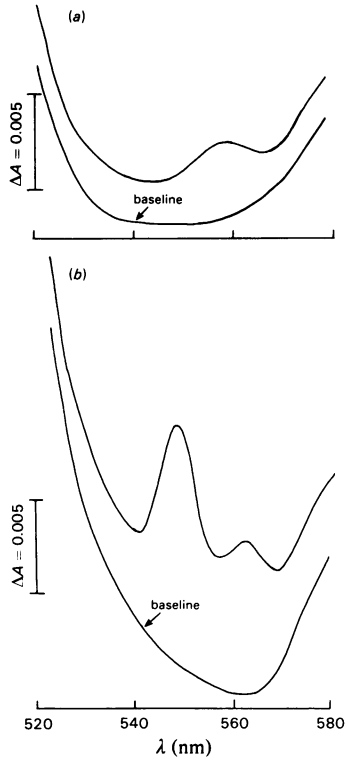

Fig. 3. *Low-potential cytochromes in Phormidium laminosum*

Difference spectra were recorded under anaerobic conditions, in a medium containing 0.5 M-sorbitol and 10 mM-$MgCl_2$ in 100 mM-Mes/NaOH buffer, pH 6.0, and membrane fragments equivalent to 100 μg of chlorophyll *a*/ml. The concentrations of menadiol and 1,4-benzoquinol were both 1 mM. $Na_2S_2O_4$ was added as a few grains of the solid. (*a*) Menadiol − 1,4-benzoquinol difference spectrum. (*b*) $Na_2S_2O_4$ − menadiol difference spectrum.

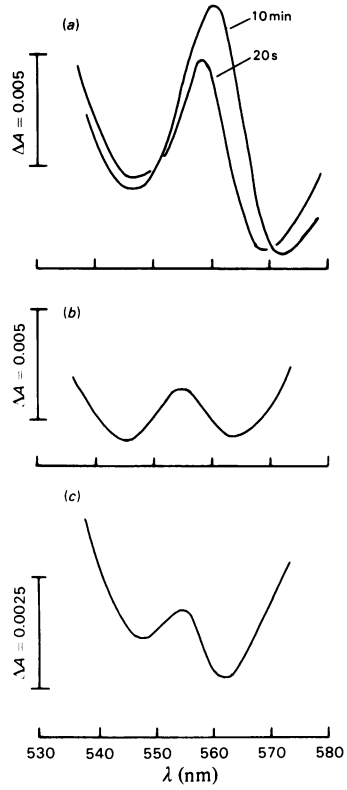

Fig. 4. *Room-temperature difference spectra of Phormidium laminosum membrane fragments after freezing, thawing and centrifuging in 'high-salt' buffer*

Membrane fragments were resuspended in 0.4 M-NaCl in 10 mM-potassium phosphate buffer, pH 7.0, then frozen at −80°C for 30 min in an ethanol/solid CO_2 bath, thawed at 25°C and centrifuged at 27 000 *g* for 20 min to pellet the fragments. The cycle of freezing, thawing and centrifuging was carried out ten times, and then the final pellet was prepared for cytochrome assays as described in Fig. 1. The final concentration of chlorophyll *a* was 85 μg/ml. (*a*) $Na_2S_2O_4$ − 1,4-benzoquinol difference spectrum. Spectra were recorded 20 s and 10 min after addition of $Na_2S_2O_4$ to the sample cuvette. (*b*) 1,4-Benzoquinol − $K_3Fe(CN)_6$ difference spectrum. (*c*) 1,4-Benzoquinol − $K_3Fe(CN)_6$ [+2% (w/v) Triton X-100] difference spectrum.

freezing and thawing, though the amounts found varied rather widely, and in some cases very little plastocyanin could be detected. *P. laminosum* plastocyanin had a broad absorbance peak at 597 nm in the oxidized form, similar to higher-plant plastocyanin (Katoh *et al.*, 1961).

Very small amounts of a basic soluble cytochrome with an α-band at 553 nm could also be extracted from spheroplasts. This cytochrome was similar to the cytochrome *c*-554 reported previously in other strains of blue–green algae (Biggins, 1967*b*; Holton & Myers, 1967; Ambler & Bartsch, 1975; Aitken, 1977).

Pigment P-700. Pigment P-700, the reaction centre of Photosystem 1, could be observed in the ascorbate-reduced − ferricyanide-oxidized difference spectrum of *P. laminosum* membrane fragments. Membrane fragments contained about 1 nmol of Pigment P-700 per 150–180 nmol of chlorophyll *a*. This value is high compared with higher plants (approx. 1 nmol of Pigment P-700 per 400 nmol of chlorophyll; Kok, 1957), but corresponds well with amounts measured in other blue–green algae (Ogawa *et al.*, 1969; Fujita, 1976).

Table 3. *Membrane-bound cytochromes in Phormidium laminosum*

Amounts of the cytochromes were measured from their reduced-minus-oxidized difference spectra, assuming absorption difference coefficients of $\Delta\varepsilon$ 20000 $M^{-1} \cdot cm^{-1}$. The values listed are ranges over at least three experiments. Approximate midpoint potentials were obtained by redox titration of fresh samples as described in the Materials and Methods section.

Cytochrome	Amount present (nmol/mg of chlorophyll *a*)	Approx. midpoint potential (mV)
f	1.0–1.2	+330 to +350
b-559 (high-potential)	2.9–3.6	+330 to +350
b-559 (low-potential)	0.95–1.1	0 to +100
b-563	2.0–2.5	0 to −100

Stability of O_2 evolution in P. laminosum

Effect of heat. P. laminosum spheroplasts showed considerably enhanced thermal stability compared with pea chloroplasts, pea chloroplast fragments and whole cells and membrane fragments of the 'semi-thermophilic' blue–green alga *Anacystis nidulans*. However, this stability largely disappeared on lysis of *P. laminosum* spheroplasts to form membrane fragments (Fig. 5).

Effect of detergent treatment. P. laminosum membrane fragments did show a surprising degree of resistance to detergent inhibition of O_2 evolution. The O_2-evolving apparatus of higher plants is extremely sensitive to detergents, and is completely inactivated at very low detergent/chlorophyll ratios (Vernon & Shaw, 1965). In contrast, both *Anacystis nidulans* and *P. laminosum* showed much greater resistance to the inhibitory effects of the detergent (Fig. 6). *P. laminosum* retained a significant proportion of its maximum O_2-evolution activity with the lipophilic electron acceptor 2,6-dimethyl-1,4-benzoquinone at detergent/chlorophyll ratios that have typically been used to fractionate the photosynthetic membranes of higher plants. Very low concentrations of Triton X-100 completely inhibited electron transport from water to Methyl Viologen in all the preparations, suggesting that the detergent had lysed the membranes and released soluble electron-transport components that function between the photosystems.

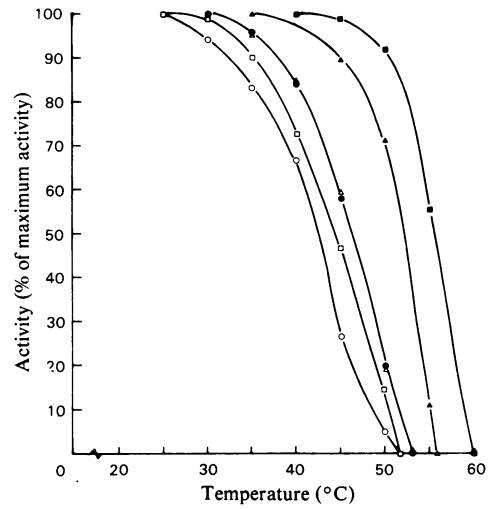

Fig. 5. *Effect of heat treatment on O_2 evolution in Phormidium laminosum, Anacystis nidulans and pea chloroplasts*

P. laminosum spheroplasts and membrane fragments, *A. nidulans* membrane fragments and pea chloroplasts were prepared as described in the Materials and Methods section. Pea chloroplast fragments were prepared by sonicating a 5 ml sample of chloroplasts (0.5 mg of chlorophyll/ml) for 60 s (Dawe Soniprobe, setting 5A). Samples (0.2 ml) of the various preparations were treated for 1 min at the temperatures shown, then cooled rapidly on ice. Rates of O_2 evolution [with 1 mM-2,6-dimethyl-1,4-benzoquinone plus 2 mM-$K_3Fe(CN)_6$] were measured at 25°C (for *P. laminosum* and *A. nidulans*) or at 20°C (for pea chloroplasts) in the oxygen electrode. Maximum rates of O_2 evolution (μmol of O_2/h per mg of chlorophyll) were: *P. laminosum* spheroplasts (■), 250; *P. laminosum* membrane fragments (●), 220; *A. nidulans* whole cells (▲), 207; *A. nidulans* membrane fragments (△), 66; pea chloroplasts (□), 120; pea chloroplast fragments (○), 90.

Discussion

The electron-transport system in the thermophilic blue–green alga *Phormidium laminosum* is similar to the system in other blue–green algae that have been investigated, showing high rates of electron transport for both the Photosystem 2 ($H_2O \rightarrow$ 2,6-dimethyl-1,4-benzoquinone) and Photosystem 1 (reduced 2,6-dichlorophenol-indophenol → Methyl Viologen) partial reactions (Arnon *et al.*, 1974; Binder *et al.*, 1976; Ono & Murata, 1978). In general, the most stable and active cell-free preparations reported have been obtained from lysozyme-sensitive strains of blue–green algae. This finding was confirmed when *P. laminosum* was compared with a number of lysozyme-insensitive thermophiles, all of which after mechanical disruption yielded preparations with low activity (A. C. Stewart, unpublished work).

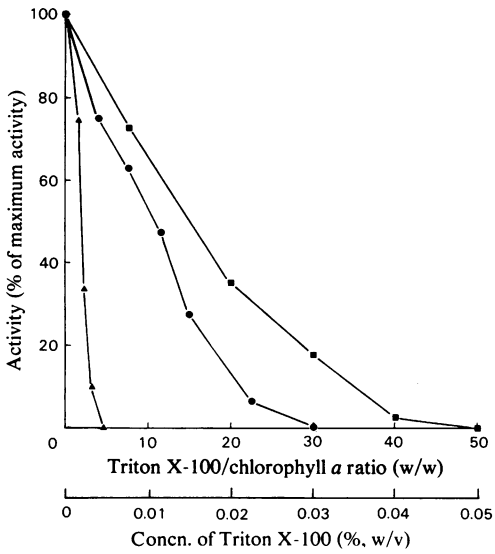

Fig. 6. *Effect of incubation with Triton X-100 on O_2 evolution by Phormidium laminosum and Anacystis nidulans membrane fragments and by pea chloroplasts*

Samples of chloroplasts or membrane fragments containing 10μg of chlorophyll were incubated for 3min in the dark in the oxygen-electrode chamber in a final volume of 1.0ml of buffer A (pH 7.5), containing various amounts of Triton X-100. After incubation, 2mM-$K_2Fe(CN)_6$ and 1mM-2,6-dimethyl-1,4-benzoquinone were added, the light was switched on and the rate of O_2 evolution was measured. Maximum rates for *P. laminosum* membrane fragments (■), *A. nidulans* membrane fragments (●) and pea chloroplasts (▲) were as listed in the legend to Fig. 5.

O_2-evolution capacity in *P. laminosum* membrane fragments was stable provided that cations or glycerol were present in the medium, approximately 10mM of bivalent or 100mM of univalent cations preserving maximal activity. Other workers have also reported cation requirements for O_2 evolution in blue–green-algal preparations (McSwain *et al.*, 1976; Binder *et al.*, 1976), and there have been reports that high concentrations of compounds such as poly(ethylene glycol) and Ficoll can stabilize activity (Fredericks & Jagendorf, 1964; Fujita & Suzuki, 1971). The reasons for these effects are not understood. Tel-Or & Avron (1974) claimed that inactivation of washed *P. luridum* membrane fragments could be reversed by adding back a soluble 'Hill factor' isolated from the supernatant, but no evidence was obtained for such a factor in *P. laminosum*.

The components of the electron-transport chain in *P. laminosum* were similar to those in several other blue–green algae. As in *Anabaena variabilis* (Lightbody & Krogmann, 1967) and *P. luridum* (Biggins, 1967*b*), plastocyanin and cytochrome *c*-553 in *P. laminosum* were water-soluble basic proteins, in contrast with eukaryotic algae and higher plants, where both proteins (where present) are acidic. Cytochrome *c*-553 in *P. laminosum* was clearly distinguishable from another *c*-type cytochrome, which was membrane-bound and appeared to be analogous to higher-plant cytochrome *f*. Wood (1977) has suggested that all oxygenic organisms contain a true membrane-bound 'cytochrome *f*' that transfers electrons to Pigment P-700 via either plastocyanin or a soluble *c*-type cytochrome; the latter two components are proposed to be functionally interchangeable. The results of the present study are consistent with this hypothesis.

The low-potential soluble cytochrome *c*-549 found in *P. laminosum* has also been observed in other blue–green algae (Biggins, 1967*b*; Holton & Myers, 1967; Pulich, 1977), but no analogous component is known in photosynthetic membranes of eukaryotic algae or higher plants. In view of the extremely low midpoint potential of this cytochrome, its function in photosynthesis is uncertain.

The amounts and midpoint potentials of the membrane-bound *b*-type cytochromes in *P. laminosum* were very similar to values reported for these cytochromes in higher plants (reviewed by Cramer & Horton, 1977). Although the photosynthetic functions of these cytochromes are still the subject of controversy, it appears that they are universally present in oxygenic organisms, including several other strains of blue–green algae (Biggins, 1967*b*; Knaff, 1973; Fujita, 1974; Aparicio *et al.*, 1974). As in higher plants (Cramer & Horton, 1977) and the blue–green alga *Anabaena variabilis* (Fujita, 1974), about 25% of cytochrome *b*-559 in fresh preparations of *P. laminosum* was present as a low-potential form (E'_0 = 0 to +100mV) not reducible by 1,4-benzoquinol. Aged or detergent-treated preparations of chloroplasts contain much larger amounts of low-potential cytochrome *b*-559 than are found in fresh preparations, at the expense of the high-potential form (E'_0 = +350 to +400mV), which is destroyed under these conditions (Bendall *et al.*, 1971). Similarly, in the present study, repeated freezing and thawing or aging at room temperature under the conditions used for redox titrations destroyed high-potential cytochrome *b*-559 in *P. laminosum*.

The preceding discussion indicates that in general terms the electron-transport system in *P. laminosum* is comparable with that of other blue–green algae and in many respects with that of higher plants. An attempt was made to discover whether the thermophily of *P. laminosum* was associated with any

enhanced stability of the alga's electron-transport system, particularly its O_2-evolving apparatus. However, the obvious thermal stability of O_2 evolution in growing cultures of *P. laminosum* is apparently not retained when the cells are broken to yield membrane fragments. It is difficult to understand why this loss of stability should occur. Enami *et al.* (1975) reported a similar phenomenon in the thermophilic red alga *Cyanidium caldarium*. In contrast, Yamaoka *et al.* (1978) have reported that in a cell-free preparation from a thermophilic blue–green alga, tentatively identified as a *Synechococcus* strain, the O_2-evolving system did exhibit the same thermophily as the original cells, and in fact showed much higher activities at temperatures near the growth temperature of the organism (50–55°C) than at room temperature.

However, despite its lack of thermal stability, the *P. laminosum* cell-free preparation did show remarkable resistance to detergent inactivation of O_2 evolution, a criterion that is likely to be of greater significance if an active O_2-evolving particle is to be obtained by solubilization and chemical fractionation of the membranes. Arnon *et al.* (1974) found that electron transport from water to $NADP^+$ in *Nostoc muscorum* was highly resistant to digitonin, but this was not a useful result in terms of fractionation of the photosystems, since clearly a functional link between the photosystems remained intact. In contrast, in *P. laminosum* Triton X-100 inhibited electron transport from water to Methyl Viologen at the lowest concentration tested, showing that membrane lysis had occurred, whereas O_2 evolution with the Photosystem 2 acceptor 2,6-dimethyl-1,4-benzoquinone remained active at detergent concentrations up to 10-fold greater, thus offering the possibility that non-ionic detergents might be used to separate the photosystems in *P. laminosum* with retention of activity. Short reports on the use of the detergents Nonident P40 and lauryldimethylamine oxide to achieve such a fractionation of *P. laminosum* membranes have appeared elsewhere (Stewart & Bendall, 1978, 1979).

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