Biochimica et Biophysica Acta, 423 (1976) 189–195

BBA 47032

PHOTOSYNTHETIC ELECTRON TRANSPORT, ATP SYNTHESIS AND NITROGENASE ACTIVITY IN ISOLATED HETEROCYSTS OF *ANABAENA CYLINDRICA*

E. TEL-OR and W. D. P. STEWART

Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, (U.K.)

(Received July 1st, 1975)

SUMMARY

Isolated heterocysts of the N_2-fixing blue-green alga *Anabaena cylindrica* contain the Photosystem I components *P*-700, bound and soluble ferredoxins and ferredoxin-NADP reductase. They also show Photosystem I activity being able to photoreduce both methylviologen and NADP when ascorbate+dichlorophenol-indophenol acts as reductant. They photophosphorylate (64 μmol ATP produced/mg chlorophyll *a*/h) and carry out oxidative phosphorylation (8.7 μmol ATP produced/mg chlorophyll *a*/h). Ninety per cent of the total cell-free extract nitrogenase activity is located in the heterocyst fraction of aerobic cultures.

INTRODUCTION

Most blue-green algae which fix N_2 in air possess characteristic cells called heterocysts. Such heterocysts arise by differentiation from vegetative cells and the fully differentiated heterocysts show many morphological, physiological and biochemical features not associated with the vegetative cells from which they are derived (see ref. 1). Among the most striking biochemical changes observed on heterocyst differentiation is the loss of certain photosynthetic pigments [2–4], their capacity to evolve O_2 [5] and their capacity to fix CO_2 [6–8]. There is also evidence, much of it indirect, that heterocysts of photosynthesising aerobically grown cultures fix N_2 whereas the vegetative cells do not [9–11]. In this paper, we present data obtained using isolated heterocysts, which show that these cells have an active Photosystem I electron transport system, that they carry out cyclic photophosphorylation, and that virtually all of the measurable cell-free extract nitrogenase activity is associated with the heterocysts of aerobically grown cultures of *A. cylindrica*.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; Cl_2Ind, 2,6-dichlorophenol-indophenol; HEPES, N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

MATERIALS AND METHODS

Organism. Anabaena cylindrica (Cambridge Culture Collection No. 1403/2a) was grown in axenic continuous culture in the nitrogen-free medium of Allen and Arnon [12] at 26 °C and 3000 lux.

Preparation of isolated heterocysts and vegetative cell fractions. Material from the continuous culture was harvested by centrifugation and resuspended in mannitol-HEPES buffer (0.5 M+30 mM, respectively), pH 7.5. Lysozyme (1 mg/ml) was added and the suspension incubated at 37 °C for 30 min, followed by centrifugation at $1000 \times g$ for 10 min. The pellet was washed in HEPES buffer (30 mM, pH 7.5) and then passed twice through a Yeda press (Yeda Scientific Instruments, Rehovot, Israel) at 1500 pounds/inch^2 under argon. The resultant suspension was diluted 3–5 fold with HEPES buffer (30 mM, pH 7.5) to which EDTA (1 mM) was added. After centrifugation at $500–1000 \times g$ for 10 min, as required, to retain the vegetative cell fragments in the supernatant and the heterocysts in the pellet, the latter was washed 2–5 times with HEPES buffer+EDTA as above and finally resuspended in a minimal volume of HEPES buffer. Such preparations were > 95 % pure heterocysts when viewed under the light microscope. The isolated heterocysts used in tests for nitrogenase activity were twice frozen and thawed rapidly, prior to the addition of the reaction mixture. In phosphorylation and electron transport studies untreated heterocysts, or heterocysts ruptured at 16 000 pounds/inch^2 by French pressure cell treatment, were used. The material used in the photosynthetic studies was prepared at 4 °C; that used in nitrogenase studies was prepared at room temperature, except for the freezing and thawing step to rupture the heterocyst membranes.

Electron transport measurements. The Mehler reaction was measured as O_2 consumption by photoreduced methylviologen when ascorbate+Cl_2Ind was supplied [13]. A Rank type O_2 electrode was used. NADP, cytochrome *c* and Cl_2Ind reductions were measured in a Unicam SP1800 spectrophotometer at 350 nm, 500 nm and 600 nm, respectively.

Phosphorylation measurements. ATP^{32} formation was measured according to the method of Avron [14]. In these experiments an ATP trapping system containing glucose and hexokinase was used to convert the ATP formed to the more stable glucose 6-phosphate, prior to assay.

C_2H_2 reduction assays. The method of Stewart et al. [15] was used.

Protein determinations. These were determined according to Lowry et al. [16] using bovine serum albumin as standard.

Chlorophyll a determinations. The spectrophotometric method of Mackinney [17] was used.

Chemicals. Lysozyme, hexokinase, creatine phosphokinase, creatine phosphate, ADP, phenazine methosulphate and HEPES were purchased from Sigma Ltd., London. ATP, NADP, NADPH and horse heart cytochrome *c* were purchased from Boehringer GmbH, Mannheim, Germany. All other chemicals were used at the purest grade commercially available from British Drug Houses Ltd., Poole, U.K. ^{32}P was obtained as orthophosphate from the Radiochemical Centre, Amersham, U.K.

RESULTS

Light-driven electron transport in preparations of isolated heterocysts

Initial studies showed, as other workers [5] had found, that preparations of intact or broken heterocysts do not evolve O_2 in the light, even in the presence of Cl_2Ind, a known acceptor of electrons from the photolysis of water via Photosystem II [13]. Electron paramagnetic resonance studies carried out in collaboration with R. Cammack and D. O. Hall of King's College, London, did show, however, photo-oxidation and reduction of *P*-700, a component previously shown to be present in isolated heterocysts by Donze et al. [18] and of membrane bound ferredoxin. These data will be presented in detail elsewhere. The presence of an active Photosystem I was demonstrated in several experiments.

Table I shows that when reduced Cl_2Ind, which donates electrons on the reducing side of *P*-700, is added to preparations of isolated heterocysts or vegetative cell fragments, photoreduction of methylviologen occurs. With the heterocyst preparations, however, good activity is obtained only when these cells are ruptured to allow penetration of the reactants to the active sites within the heterocysts. There is a negligible increase in activity when vegetative cell fragments are ruptured by French press treatment, indicating that the increased activity on rupturing the heterocysts is not due to breakage of any contaminating vegetative cell material. The data support those of Wolk and Simon [3].

Data showing NADP photoreduction by preparations of isolated heterocysts are presented in Table II. Using ascorbate+Cl_2Ind as electron-donating system, photoreduction occurs in the absence of added ferredoxin and ferredoxin-NADP reductase (Table II, Reaction 1) which indicates that sufficient endogenous concentrations of these components are present in heterocysts to enable electron transport to NADP to occur. The presence of ferredoxin-NADP reductase and soluble ferredoxin in the isolated heterocyst preparations was confirmed by showing, first, that heterocyst extracts could reduce Cl_2Ind on adding NADPH in the dark (a reaction dependent on ferredoxin-NADP reductase) (Table II, Reaction 2) and second, that they reduced

TABLE I

PHOTOREDUCTION OF METHYLVIOLOGEN BY ISOLATED HETEROCYSTS OF *ANABAENA CYLINDRICA* SUPPLIED WITH ASCORBATE+Cl_2Ind

The reaction mixture contained in 3 ml of 30 mM HEPES buffer, pH 7.5: 6.7 mM ascorbate+$5 \cdot 10^{-5}$ M Cl_2Ind as electron donor and $5 \cdot 10^{-5}$ M methylviologen in the presence of 2.5 mM NaN_3. 15–40 μg chlorophyll *a* were present per sample. The light intensity was $7 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} and the temperature 25°C. Each value is the mean of duplicate determinations.

Material	μmol O_2 consumed · mg chlorophyll $a^{-1} \cdot h^{-1}$	Relative activity
Vegetative cell fragments	588	100
Intact isolated heterocysts	66	11
Ruptured isolated heterocysts (French press)	252	43
Ruptured vegetative cell fragments (French press)	711	121

TABLE II

FERREDOXIN AND FERREDOXIN-NADP REDUCTASE MEDIATED REACTIONS IN ISOLATED HETEROCYSTS OF *ANABAENA CYLINDRICA*

The activities were measured in a reaction mixture containing in 3 ml, 30 mM HEPES buffer, pH 7.5 and broken heterocysts containing 15–40 μg chlorophyll *a*. The temperature was 25 °C. The NADP photoreaction mixture also contained: 6.7 mM ascorbate; 0.05 mM Cl_2Ind and 1 mM NADP, in the light ($7 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}). Cl_2Ind and cytochrome *c* reaction mixtures contained: 33 μM NADPH and either 33 μM cytochrome *c* or 20 μM Cl_2Ind. Each value is the mean of duplicate determinations.

Reaction	Activity (μmol reductant formed $\cdot$ mg chlorophyll $a^{-1} \cdot h^{-1}$)
1. Reduced $Cl_2Ind \rightarrow$ NADP photoreduction	13
2. NADPH $\rightarrow$ Cl_2Ind	95
3. NADPH $\rightarrow$ cytochrome *c*	59

horse-heart cytochrome *c* in the dark when NADPH was added (a reaction requiring both ferredoxin-NADP-reductase and ferredoxin) (Table II, Reaction 3).

Thus, photosynthetic electron transport from the reducing side of *P*-700 to ferredoxin and NADP occurs in isolated heterocysts. Such results obtained with preparations of isolated heterocysts extend the findings of Bothe [19], who used higher plant chloroplast material, and Smith et al. [20], who used lamellar fragments of *A. cylindrica*, to show that ferredoxin, ferredoxin-NADP reductase and NADP can donate electrons to *A. cylindrica* nitrogenase in the light.

Phosphorylating activities of isolated heterocysts

Indirect evidence of photophosphorylation by isolated heterocysts of *A. cylindrica* has been obtained by Wolk and Wojciuch [21], while Scott and Fay [22],

TABLE III

PHOSPHORYLATING ACTIVITIES (ΔATP^{32} FORMATION) OF ISOLATED HETEROCYSTS OF *ANABAENA CYLINDRICA*

The reaction mixture contained in 3 ml, (mM): KCl, 100; ascorbate, 6.7; HEPES, pH 7.5, 30; ADP, 3.3; phosphate, 6.7 containing $1–3 \cdot 10^6$ cpm ^{32}P; $MgCl_2$, 2; phenazine methosulphate, 0.05; 15–40 μg chl *a*. The ATP-trapping system contained hexokinase (Sigma type IV, 4 units) and 6 mM glucose. The light intensity was $7 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} and the temperature was 25 °C. Each value is the mean of duplicate determinations.

Material	Conditions	μmol ATP formed $\cdot$ mg chlorophyll $a^{-1} \cdot h^{-1}$
Isolated heterocysts	Light/air	0
	Light/argon	9.1
	Dark/air – argon	0
Ruptured isolated heterocysts (French press)	Light/air	63.8
	Light/argon	61.0
	Dark/air – argon	8.7

in a preliminary report, quote rates of photophosphorylation of 19–27 μmol ATP formed/mg chlorophyll *a*/h by isolated heterocysts of *Anabaena variabilis*. There is also evidence from whole cell studies [23, 24] that cyclic photophosphorylation may be the main source of ATP for nitrogenase. In this study we measured phosphorylation by isolated heterocysts directly using ^{32}P as tracer and, as stated in the Materials and Methods section, ATP^{32} synthesis was confirmed using an ATP trapping system in which glucose and hexokinase were used to convert the ATP formed to glucose 6-phosphate prior to assay.

Data obtained using vegetative cell fragments and heterocyst preparations of aerobically grown cultures (Table III) show that there was little, or no, phenazine methosulphate mediated photophosphorylation by the heterocysts until they were ruptured. Photophosphorylation by vegetative cell fragments occurred, but did not increase significantly on French press treatment (data not shown). No evidence of photophosphorylation coupled to methylviologen photoreduction was obtained. Oxidative phosphorylation by extracts of isolated heterocysts was also demonstrated, this activity representing 13–14 % of the light rate. Thus, ATP and reductant, both of which are essential for nitrogenase activity in *A. cylindrica* [19, 25, 26] can be provided via the Photosystem I machinery present in the heterocysts of this alga.

Nitrogenase activity of isolated heterocysts

There are conflicting views on the role which the heterocysts of aerobically grown blue-green algae play in N_2-fixation [9–11, 27, 28]. We have reinvestigated the distribution of nitrogenase activity between heterocysts and vegetative cells of aerobi-

TABLE IV

DISTRIBUTION OF NITROGENASE ACTIVITY BETWEEN VEGETATIVE CELLS AND HETEROCYSTS OF AEROBICALLY GROWN *ANABAENA CYLINDRICA*

The reaction mixture contained (mM): ATP, 5; $MgCl_2$, 5; creatine phosphate, 8; $Na_2S_2O_4$, 5; phosphocreatine kinase, 200 μg. Protein, 1–2 mg. The reaction was carried out under A/C_2H_2 (90/10, v/v) for 15 min at a light intensity of $2.2 \cdot 10^3$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1} at 25 °C. Specific activity is calculated as nmol $C_2H_4 \cdot$ mg protein$^{-1} \cdot$ min^{-1}; total activity is calculated as nmol $C_2H_4 \cdot$ min^{-1}. Each value is the mean of duplicate determinations.

Specific activity of intact filaments prior to breakage	0.194
Total activity of intact filaments prior to breakage	36.00
Specific activity of ruptured isolated heterocysts	0.680
Total activity of ruptured isolated heterocysts	16.60
Specific activity of vegetative cell material	0.008
Total activity of vegetative cell material	1.76
Heterocyst activity as % of intact filament activity	45
Heterocyst activity as % of total cell-free activity	90

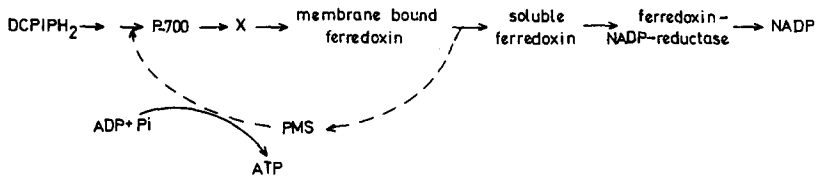

Fig. 1. A tentative scheme involving some electron transport components of isolated heterocysts of *A. cylindrica*.

cally grown *A. cylindrica*, after separating heterocyst and vegetative cell fractions as described in the Materials and Methods section. The technique for separating the fractions, which involves the use of lysozyme coupled with Yeda pressure cell treatment, is much more gentle than any hitherto used.

Table IV presents data on C_2H_2 reduction by various algal fractions. The specific activity of the algal suspension prior to breakage was 0.194 nmol C_2H_4/mg protein/min giving a total activity in the intact filaments of 36 nmol/min. After lysozyme and Yeda press treatment there was a 49 % loss in activity and of the activity remaining (18.36 nmol/min) 98 % was pelleted on subsequent centrifugation. On separation of this pellet into heterocyst and vegetative cell fractions it was found that the specific activity of the ruptured isolated heterocysts was 0.680 nmol C_2H_4/mg protein/min. This value is 3.5 times higher than the specific activity of the intact filaments and is 85 times higher than the specific activity of the vegetative cell fraction. The total activity in the heterocyst fraction (16.6 nmol C_2H_4/min) accounts for 90 % of the total cell-free activity.

An obvious interpretation of such findings, together with those of Fleming and Haselkorn [10, 11], who obtained evidence using gel electrophoretic techniques that nitrogenase proteins were present in the heterocysts but not in vegetative cells of aerobically grown *Nostoc muscorum*, and the various other lines of indirect evidence [29], is that the heterocysts are the main or sole sites of nitrogenase activity in aerobic cultures of *A. cylindrica*. However, even in studies of this type the possibility cannot be ruled out of a selective loss of vegetative cell nitrogenase [28], although no evidence that this was occurring was obtained.

The above results obtained with isolated heterocysts thus provide evidence in support of the view that the heterocyst is a highly specialised cell whose prime function is to fix N_2, and that while its photosynthetic machinery is modified so that photosynthetically evolved O_2 (which would inhibit nitrogenase) is not produced, ATP from cyclic photophosphorylation and a route of electron transport from fixed carbon compounds to ferredoxin and NADP via Photosystem I is available [30]. Indeed, the rates of cyclic photophosphorylation, and of photoreduction of ferredoxin and NADP observed in vitro (Tables II–III) are sufficient to support not only the in vitro rates (Tables IV) but also the reported in vivo rates of nitrogenase activity in *A. cylindrica* [31]. The overall interactions based on our data are summarised in Fig. 1.

ACKNOWLEDGEMENTS

This work was made possible by research support to W.D.P.S. from the Science Research Council, the Natural Environment Research Council and the

Royal Society. We thank Dr. G. A. Codd and Mr. P. J. Bottomley for help with various aspects of the work.

REFERENCES

1 Fogg, G. E., Stewart, W. D. P., Fay, P. and Walsby, A. E. (1973) The blue-green algae, pp. 459, Academic Press, London
2 Fay, P. (1969) Arch. Mikrobiol. 67, 62–70
3 Wolk, C. P. and Simon, R. D. (1969) Planta (Berlin) 86, 92–97
4 Thomas, J. (1970) Nature (Lond.) 228, 181–183
5 Carr, N. G. and Bradley, S. (1973). Symp. Soc. Gen. Microbiol. 23, 161–188
6 Fay, P. and Walsby, A. E. (1966) Nature (Lond.) 209, 94–95
7 Wolk, C. P. (1968) J. Bacteriol. 96, 2138–2143
8 Stewart, W. D. P., Haystead, A. and Pearson, H. W. (1969). Nature (Lond.) 224, 226–228
9 Stewart, W. D. P. (1973) Annu. Rev. Microbiol. 27, 283–316
10 Fleming, H. and Haselkorn, R. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2727–2731
11 Fleming, H. and Haselkorn, R. (1974) Cell 3, 159–170
12 Allen, M. B. and Arnon, D. I. (1955) Plant Physiol. 30, 366–372
13 Vernon, L. P. and Zaugg, W. S. (1960) J. Biol. Chem. 235, 2728–2733
14 Avron, M. (1961) Anal. Biochem. 2, 535–543
15 Stewart, W. D. P., Fitzgerald, G. P. and Burris, R. H. (1967) Proc. Natl. Acad. Sci. U.S. 58, 2071–2078
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
17 Mackinney, G. (1941) J. Biol. Chem. 140, 315–322
18 Donze, M., Haveman, J. and Schiereck, P. (1972) Biochim. Biophys. Acta 256, 157–161
19 Bothe, H. (1970) Ber. Deut. Botan. Ges. 83, 421–432
20 Smith, R. V., Noy, R. J. and Evans, M. C. W. (1971) Biochim. Biophys. Acta 253, 104–109
21 Wolk, C. P. and Wojciuch, E. (1971) Planta (Berlin) 97, 126–134
22 Scott, W. E. and Fay, P. (1972) Brit. Phycol. J. 7, 283–284
23 Cox, R. M. and Fay, P. (1969) Proc. Roy. Soc. B. 172, 357–366
24 Stewart, W. D. P. and Bottomley, P. J. (1975) In Proc. Internat. Symp. on N_2-Fixation (W. E. Newton and C. J. Nyman, eds), Washington State Univ. Press, Pullman (in press)
25 Smith, R. V. and Evans, M. C. W. (1970) Nature (Lond.) 225, 1253–1254
26 Haystead, A., Robinson, R. and Stewart, W. D. P. (1970) Arch. Mikrobiol. 74, 235–243
27 Ohmori, M. and Hattori, A. (1971) Plant Cell Physiol. 12, 961–967
28 Wolk, C. P., Austin, S. M., Bortins, J. and Galonsky, A. (1974) J. Cell Biol. 61, 440–458
29 Stewart, W. D. P. (1974) The biology of nitrogen fixation, (A. Quispel, ed.), pp. 202–237, North Holland Publishing Co., Amsterdam
30 Donze, M., Raat, A. J. P. and Van Gorkom, H. J. (1974) Plant Sci. Lett. 3, 35–41
31 Stewart, W. D. P., Fitzgerald, G. P. and Burris, R. H. (1968) Arch. Mikrobiol. 62, 336–348