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COMPONENTS AND ACTIVITY OF THE PHOTOSYNTHETIC ELECTRON TRANSPORT SYSTEM OF INTACT HETEROCYSTS ISOLATED FROM THE BLUE-GREEN ALGA *NOSTOC MUSCORUM*

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Summary

Heterocysts of the blue-gree alga *Nostoc muscorum* have been isolated by prolonged treatment with lysozyme. Quantitative data are presented which show the occurrence of cytochromes *c*-553, *f*-557 and *b*-563 in heterocysts in amounts comparable to vegetative cells. Particularly the content of the water-soluble cytochrome *c*-553 can be used to evaluate the intactness of a heterocyst preparation. Cytochrome *f*-557 has been partially purified and found to be a *c*-type cytochrome corresponding to cytochrome *f* of higher plants and other algae. Cytochrome *b*-559 is present in vegetative cells but not in heterocysts. The content of plastoquinone in heterocysts is reduced to 42% of the amount present in vegetative cells. These data suggest a degradation of Photosystem II during heterocyst differentiation.

Measurements of photosynthetic electron transport in heterocysts proved the inability of the photosynthetic apparatus to carry out electron transport with electrons donated by water or diphenylcarbazide. In Tris-washed thylakoids from vegetative cells, however, diphenylcarbazide can act as an electron donor to Photosystem II.

Introduction

Many filamentous blue-green algae have heterocysts which are specialized cells morphologically and physiologically different from vegetative cells [1]. Most of the nitrogen fixation occurs in heterocysts, and they have developed

Abbreviations: DCMU, *N*-(3,4-dichlorophenyl)-*N'*,*N'*-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)-methylglycine; Chl, chlorophyll.

particular mechanisms to protect the oxygen-sensitive nitrogenase. Heterocysts are enclosed by a thick outer envelope reducing the diffusion of oxygen. Photosystem II is not functional and oxygen is not evolved during photosynthesis. Moreover, heterocysts are apparently devoid of phycobiliproteins [2] which probably are the accessory pigments of Photosystem II.

According to the only comprehensive study on photosystems in heterocysts so far published [3], it is assumed that heterocysts contain both photosystems. Photosystem II is thought to be inactivated by a significant decrease in the Mn^{2+} level of heterocysts. No quantitative data are reported so far comparing the amounts of photosynthetic electron transport carriers of heterocysts and vegetative cells. In the present study, the occurrence and quantitative distribution of cytochromes, plastoquinone, and *P*-700 were investigated in heterocysts and filaments of the blue-green alga, *Nostoc muscorum*. Care was taken to prevent leakage of soluble components from heterocysts during their isolation. Therefore, a gentle isolation procedure was applied involving enzymic digestion of vegetative cells. Furthermore, the capability of the photosynthetic apparatus of heterocysts to perform several electron transport reactions was studied in comparison with filamentous cells.

Materials and Methods

Cultivation of algae. Nostoc muscorum (strain No. 7119) was axenically grown as described [4] in a nitrogen-free medium according to [5]. The cultures were continuously illuminated by fluorescent white light (7 W/m^2), bubbled with air enriched with 5% CO_2, and held at 26—28° C. Algae were harvested at the late logarithmic phase by centrifugation (5 min at 4000 × *g*).

Isolation of heterocysts. Filaments were suspended in a medium containing 50 mM Hepes/NaOH, pH 7.2; 10 mM NaCl; and 0.4 M sucrose. The chlorophyll concentration of this suspension was 200—400 μg Chl *a*/ml. After 30 min of equilibration with the buffer, lysozyme was added (3 mg/ml) and the suspension was incubated at 30° C under vigorous stirring for 20 h. Thereafter, lysis of vegetative cells was complete and heterocysts could be isolated by several low-speed centrifugations (2 min at 1000 × *g*). The degree of purity of the heterocyst suspension as judged by a light microscope was 90—95%. Intact heterocysts were stored in the Hepes/NaCl/sucrose buffer without lysozyme at 0° C for immediate use or quickly frozen in liquid nitrogen and stored at —20° C for some quantitative determinations.

Preparation of thylakoids. Whole filaments or isolated intact heterocysts were mixed with an equal volume of glass beads (0.5 mm diameter) and shaken for 2 min in a cell homogenizer (Vibrogen Zellmühle). The resulting cell homogenate was filtered through a glass suction filter to remove the glass beads and centrifuged for 5 min at 5000 × *g* to remove intact cells and larger cell debris. The supernatant was centrifuged for 60 min at 48 000 × *g* and the resulting pellet carefully suspended in 50 mM Tris/HCl, pH 7.8, containing 10 mM $MgCl_2$. These suspensions were used for measurement of electron transport and difference spectra. Thylakoids of vegetative cells used for difference spectra were additionally washed several times with Tris-buffer until the supernatant appeared virtually colourless (removal of phycocyanin) to facilitate

the determination of cytochromes by difference absorption spectroscopy.

Determination of cytochromes. Thylakoid suspensions of heterocysts and filaments with a chlorophyll concentration of 40—80 μg Chl *a*/ml were used for difference spectra (Shimadzu spectrophotometer UV 300 equipped with a baseline corrector DBC-2). After recording a baseline, ferricyanide, hydroquinone, ascorbate, or dithionite were added as indicated (Fig. 1).

For determination of the cytochrome *f*-557 content a line was drawn from the peak maximum at 557 nm to the line connecting the isosbestic points at 545 nm and 563 nm, giving a value for the absorbance difference. For quantitative evaluation the differential millimolar extinction coefficient 21.5 $mM^{-1} \cdot cm^{-1}$ was used. Isosbestic points and the extinction coefficient were obtained from a pure preparation of cytochrome *f*-557 from *Spirulina* (Bohme, Pelzer and Böger [18]). The content of cytochromes *b*-559 and *b*-563 was determined according to [6] using $\epsilon^{\Delta}_{559-570} = 15$ and $\epsilon^{\Delta}_{563-570} = 14$ ($mM^{-1} \cdot cm^{-1}$).

Soluble cytochrome *c*-553 was determined in the supernatants after cell breakage. Quantitative evaluation followed the procedure described for cytochrome *f*-557 using the isosbestic points 541 nm and 561 nm and $\epsilon^{\Delta}_{553} = 17$ ($mM^{-1} \cdot cm^{-1}$) from *Scenedesmus* [7].

Determination of P-700. Thylakoids of filaments and heterocysts were suspended in 50 mM Tris/HCl, pH 7.8, containing 0.17% Triton X-100 and 0.17 μM ferricyanide (chlorophyll concentration 10 μg/ml). After recording a baseline, hydroquinone was added (0.34 μM) to one cuvette and the difference was measured. The content of *P*-700 was calculated using the differential millimolar extinction coefficient 64 $mM^{-1} \cdot cm^{-1}$ [8].

Determination of plastoquinone. Thylakoids of filaments and heterocysts (total chlorophyll content 3 mg) were extracted by the isopropanol/heptane/water method as described by Barr and Crane [9]. Separation of quinones was achieved by thin-layer chromatography. The plastoquinone band was scraped off, dissolved in ethanol, and spectra were recorded with a Shimadzu spectrophotometer UV 360. Calculation of the plastoquinone content was done by evaluating the oxidized minus $NaBH_4$-reduced difference spectra using the differential millimolar extinction coefficient 15 ($mM^{-1} \cdot cm^{-1}$) [9].

Measurement of electron transport rates. All measurements were performed with a YSI oxygen monitor mod. 53 at 25° C (for details see Table II).

Tris-washed thylakoids were prepared by incubating thylakoids (chlorophyll-*a* concentration 200 μg/ml) in 0.8 M Tris/HCl, pH 8.8, at 0° C for 30 min according to Yamashita and Tomita [10].

Results

One of the main objects of the present study was to isolate intact heterocysts, which in addition to their undamaged appearance in the light microscope should contain soluble cytochrome *c*-553. Of all the methods tried, only a long-time incubation of filaments with lysozyme (to destroy vegetative cells) followed by several low-speed centrifugations (to collect heterocysts) proved to be satisfactory. Short-time lysozyme incubation with subsequent ultrasonic or Yeda-press treatments resulted in a loss of cytochrome *c*-553 as did French-press treatment. Short-time lysozyme incubation followed by mild sonication

in a microsonic cleaning bath as reported for *Anabaena variabilis* [11] failed to destroy vegetative cells of *Nostoc muscorum*, presumably because of the more rigid cells wall of this species.

Table I summarizes the data found for the content of cytochromes, plastoquinone, and *P*-700 in filaments and heterocysts. Related to chlorophyll-*a* content, heterocysts appear to have a higher concentration of these redox carriers than filaments (Table I). This is deceptive, however because of the generally decreased chlorophyll-*a* content of heterocysts [2]. To allow a comparison between the photosynthetic apparatus of filaments and heterocysts, the redox carrier present in least amount (= cytochrome *f*-557) was deliberately set = 1, and the amount of the other redox carriers was related to cytochrome *f*-557 (Table I). *P*-700, the reaction center chlorophyll, cannot be taken as a reference, since it has been shown that its content may vary considerably depending on the light conditions of growth [12].

Difference spectra of the supernatants of broken filaments and heterocysts did not reveal significant differences of the cytochrome *c*-553 content. The small discrepancy actually occurring may be attributed to some leakage of cytochrome *c*-553 out of heterocysts during lysozyme incubation.

Nostoc muscorum obviously belongs to those algae which can only synthesize soluble *c*-type cytochrome but no plastocyanin (compare Refs. 13 and 14). Variation of the cupric ion concentration in the culture medium of up to 1 μM had no influence on the content of soluble cytochrome *c*-553 in filaments and heterocysts nor was plastocyanin detectable by difference spectroscopy according to Bohner and Böger [15] (compare also Refs. 16 and 17).

In the difference spectra of thylakoids of filaments three membrane-bound cytochromes can be distinguished (Fig. 1): cytochrome *f*-557 (hydroquinone-reducible), cytochrome *b*-559 (ascorbate-reducible), and cytochrome *b*-563 (dithionite-reducible). Thylakoids of heterocysts lack cytochrome *b*-559 but do contain cytochromes *b*-563 and *f*-557 in amounts comparable to thylakoids of filaments (Table I).

Cytochrome *f*-557 has been partially purified according to Böhme et al. [18], to characterize it as a membrane-bound *c*-type cytochrome with a γ-band at 422 nm and an α-band at 557 nm. The shoulder at 550 nm in the difference

TABLE I

CONTENT OF PHOTOSYNTHETIC REDOX CARRIER IN HETEROCYSTS AND FILAMENTS OF *NOSTOC MUSCORUM*

	Molar ratio of chlorophyll *a* to redox carrier		Molar ratio of redox carrier to cytochrome *f*-557	
	Filaments	Heterocysts	Filaments	Heterocysts
Cytochrome *c*-553	200	90	3.2	2.5
Cytochrome *f*-557	637	223	1	1
Cytochrome *b*-559	600	—	1	—
Cytochrome *b*-563	300	95	2.1	2.3
P-700	280	90	2.3	2.5
Plastoquinone	37	31	17.2	7.2

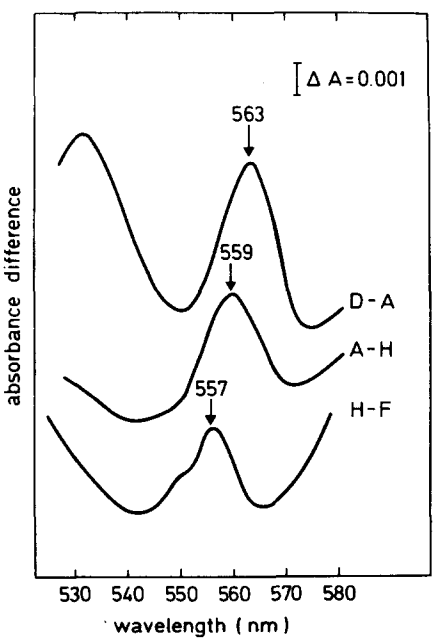
spectrum of thylakoids (Fig. 1) is part of the cytochrome *f*-557 spectrum (Fig. 2) and not due to superimposition by an additional cytochrome *c*-549 [19].

Both heterocysts and filaments have a high concentration of *P*-700. About 2.5 mol *P*-700 per 1 mol cytochrome *f*-557 were found. This high concentration of *P*-700 seems to be typical for plants grown under low-light conditions [12].

A significant difference is found as far as the plastoquinone content of filaments and heterocysts is concerned. 17.2 mol plastoquinone per 1 mol cytochrome *f*-557 are present in filaments whereas only 7.2 mol plastoquinone are found in heterocysts. So the plastoquinone pool in heterocysts is reduced to 42% in comparison to filaments.

Measurements of photosynthetic electron transport rates of thylakoids confirmed earlier reports [20]. Heterocysts cannot carry out Photosystem-II mediated electron transport from water to an artificial acceptor such as ferricyanide or methyl viologen. Electron transport from the artificial donor diaminodurene to methyl viologen is, however, possible indicating that Photosystem I is functional in heterocysts (Table II).

It was of special interest to investigate whether the inability to evolve oxygen was due to an inactivation of the water-splitting system of Photosystem II caused by Mn^{2+} deficiency in heterocysts [3], or whether parts of Photosystem II were inoperative. The date in Table II show that Tris-washed thyla-



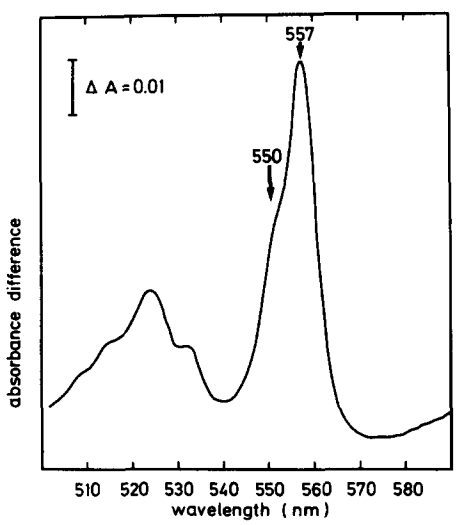


Fig. 1. Difference spectra (reduced minus oxidized) of thylakoids from filaments of *Nostoc muscorum*. Chlorophyll *a* concentration 69 μg/ml. Chemicals were added as a solid. F, ferricyanide; H, hydroquinone; A, ascorbate; D, dithionite.

Fig. 2. Difference spectrum (hydroquinone-reduced minus ferricyanide-oxidized) of partially purified cytochrome *f*-557 from *Nostoc muscorum*.

TABLE II

PHOTOSYNTHETIC O_2 EVOLUTION OR UPTAKE (± μmol O_2/mg Chl *a*/h) IN THYLAKOIDS OF HETEROCYSTS AND FILAMENTS OF *NOSTOC MUSCORUM*

The reaction mixture contained: 20 mM Tricine/NaOH, pH 7.8; 10 mM $MgCl_2$; 5 mM sodium-potassium phosphate (for O_2-uptake measurements additionally 0.1 mM NaN_3 and 1 mM KCN); and the following additions: methyl viologen (0.1 mM), diaminodurene (DAD, 0.1 mM), ascorbate (5 mM), ferricyanide (1 mM), diphenylcarbazide (DPC, 2.5 mM), DCMU ($5 \cdot 10^{-6}$ M). The chlorophyll concentration was 20 μg Chl *a*/ml; temperature 25°C and light intensity 1000 W/m^2 (red light, RG 610 plus heat filter KG 1 from Schott, Mainz).

Assay system	Oxygen evolution or uptake			
	Thylakoids		Tris-washed thylakoids	
	Filaments	Heterocysts	Filaments	Heterocysts
$H_2O \rightarrow$ ferricyanide	+76	0	0	0
$H_2O \rightarrow$ methyl viologen	—102	0	0	0
DAD/ascorbate → methyl viologen	—998	—651	—	—
DPC → methyl viologen	—	—	—54	0
DPC → methyl viologen + DCMU	—	—	—9	—

koids of filaments are not able to perform electron transport from water to methyl viologen because of an inactivation of their water-splitting system. However, they can perform electron transport from the artificial donor, diphenylcarbazide, to methyl viologen. This reaction is DCMU-sensitive, indicative of a Photosystem-II-mediated reaction. On the other hand, thylakoids of heterocysts cannot perform electron transport from diphenylcarbazide to methyl viologen. This suggests that the process of heterocyst differentiation with respect to the photosystems results in more than manganese deprivation only.

Discussion

This study is in agreement with earlier reports on heterocysts concerning the presence and function of Photosystem I. The quantitative data reported in this paper show that the investigated redox carriers of Photosystem I are present in about equal amounts in filaments and heterocysts. We also noted that the chlorophyll content of heterocysts is reduced by one-third compared to vegetative cells. It was shown in addition that heterocyst thylakoids lack a 100-Å particle, the Photosystem II-complex of vegetative cells [24]. Photosystem II of heterocysts is obviously degraded to a greater extent than suggested by Tel-or and Stewart [3]. There are several findings which are in contrast with those data published for *Anabaena cylindrica* [3]. Our data clearly show that heterocysts of *Nostoc muscorum* contain no cytochrome *b*-559. The data on the occurrence of cytochrome *b*-559 in heterocysts of *Anabaena* are not conclusive. Tel-Or and Stewart could not measure a room-temperature spectrum of cytochrome *b*-559. Only the spectrum at 77 K shows two bands with peaks at 558 and 561 nm. This is very likely to represent cytochrome *b*-563 alone, which shows a split α-band at low temperature [21]. The absence of cytochrome *b*-559 in heterocysts is important with respect to its close association with Photosystem II.

Contrary to Ref. 3 it was not possible, either, to demonstrate electron trans-

port in heterocysts with the artificial donor diphenylcarbazide, although it may act as a donor to Photosystem II, as demonstrated with Tris-washed thylakoids of filaments. It has been reported that electron transport from diphenylcarbazide to an artificial electron acceptor is DCMU-insensitive in thylakoid preparations of blue-green algae [22,23]. These investigations were done with *Anabaena cylindrica* and *Oscillatoria chalybea*, suggesting a different DCMU-binding site for blue-green algae. This cannot be confirmed for *Nostoc muscorum*. The plastoquinone pool in heterocysts which is reduced to 42% in comparison to filaments may be taken as additional evidence for a degraded Photosystem II in heterocysts. It is commonly thought that the plastoquinone pool is shared by both photosystems.

The existence of a membrane-bound *c*-type cytochrome with an α-band at 557 nm seems to be typical for blue-green algae, but in many cases has not been recognized. From the blue-green alga, *Spirulina*, we have obtained a pure preparation of cytochrome *f*-557, resembling in its biochemical properties those of cytochrome *f* of higher plants (compare also Ref. 18). With this finding many conclusions from studies on light-induced redox reactions of cytochromes of blue-green algae may become questionable. Although cytochrome *b*-559 can be photooxidized at 77 K in *Nostoc* [25], other data of a component, absorbing at 557—558 nm, resemble more the properties of cytochrome *f* of higher-plant chloroplasts: Firstly, low concentrations of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone, the plastoquinone antagonist, fail to inhibit photooxidation [26]; secondly, red light causes an increased oxidation in the presence of $NADP^+$ [19]. Another remarkable feature of the light-induced spectra was a pronounced shoulder at 550 nm, which was interpreted as participation of a cytochrome *c*-549 in electron transport [18,19].

The data on partial purification of this component as reported here demonstrate that the unusual absorbance characteristics of *Nostoc* cytochrome *f*-557 (with a shoulder at 550 nm) may have caused this misinterpretation of light-induced absorbance changes. Further studies will have to reexamine some of the data reported, especially concerning the role of cytochromes *b*-559 and *c*-549 in blue-green algae.

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