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Respiratory activities and aa_3-type cytochrome oxidase in plasma and thylakoid membranes from vegetative cells and heterocysts of the cyanobacterium *Anabaena* ATCC 29413

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Plasma and thylakoid membranes were separated by discontinuous sucrose density gradient centrifugation from crude membrane preparations of vegetative cells and heterocysts of the nitrogen-fixing cyanobacterium *Anabaena* ATCC 29413. Isolated and purified plasma membranes were devoid of spectroscopically detectable chlorophyll. Intact heterocysts were separated from vegetative cells by selective lysozyme degradation of the latter, followed by low-pressure French-press extrusion and differential centrifugation. Each of the four types of membrane preparation oxidized horse heart ferrocytochrome *c*, rates ranging from 35 nmol/min per mg protein (plasma membrane from vegetative cells) to 2400 nmol/min per mg protein (thylakoid membranes from heterocysts). The reactions were stimulated up to 6-fold by 0.05% (w/v) *n*-octyl glucoside; they were completely inhibited by 1.2 μM KCN and severely inhibited by CO. The cytochrome oxidase present in *n*-octylglucoside-solubilized membranes could be enriched about 10-fold by affinity chromatography on immobilized yeast cytochrome *c*. Extraction with acidic butanone-2 or acetone, followed by preparation of the alkaline pyridine ferrohemochrome derivatives, proved the presence of *a*-type cytochrome. Sodium dodecylsulfate polyacrylamide gel electropherograms of the four membrane types were characteristically different from each other. Immunoblotting of the membrane polypeptides with antisera against the aa_3-type cytochrome of *Paracoccus denitrificans* (subunits I and II and holoenzyme) and rat liver mitochondria (subunit II) gave specific and complementary cross-reaction at about 48–49 and 36 kDa (subunits I and II, respectively) irrespective of the membranes used. In addition to oxidizing ferrocytochrome *c*, the isolated membranes were also found to reduce horse heart ferricytochrome *c* to variable extents with NAD(P)H.

Introduction

Nitrogen-fixing cyanobacteria are often called the nonplus-ultra of bioenergetics. Utilizing light and water (by virtue of oxygenic, plant-type photosynthesis, otherwise not encountered among prokaryotes), carbon dioxide and dinitrogen from the atmosphere, and some ubiquitous inorganic

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; OG, *n*-octyl glucoside; Pipes, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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ions, they have already been sustaining their life processes for at least 3.2 billion years [1,2]. While it has been firmly established that cyanobacterial photosynthesis (including electron transport) follows the same mechanism as does chloroplast photosynthesis [3,4], amazingly little is known about respiratory electron transport in these unique prokaryotes [5,6]. However, as techniques to separate and isolate plasma and thylakoid membranes from cyanobacteria have become available [7–9], it is now possible to perform detailed biochemical studies on the composition, function and localization of the respiratory electron transport assemblies. Various rates of respiratory electron transport were found with isolated and purified plasma and thylakoid membranes from unicellular species [8–12], the relative shares crucially depending on growth conditions [9–11]. Basically, the chlorophyll-free plasma membranes from eight different species investigated so far were shown to be a potential site of respiratory electron transport (Refs. 8–11 and G.A. Peschek et al., unpublished results), in addition to the intracytoplasmic photosynthetic lamellae (thylakoid membranes) housing electron transport components common for both respiration and photosynthesis [13–15]. aa_3-type cytochrome *c* oxidase (EC 1.9.3.1) has been detected in most of these species [8,9,16–19]; the enzyme, however, has not yet been isolated and obtained in a pure state from any cyanobacterium. In this communication we want to show that both plasma and thylakoid membranes of nitrogen-fixing cyanobacterium *Anabaena* ATCC 29413 are competent sites of respiratory electron transport comprising, in particular, varying activities of NAD(P)H dehydrogenases and aa_3-type cytochrome oxidase. The exceedingly high respiratory activity of heterocysts, which appears to be due to a considerably more active, rather than higher concentrations of, aa_3-type cytochrome oxidase in these differentiated cells, will be discussed in terms of a possible role for the so-called respiratory protection of nitrogenase.

Materials and Methods

Axenic suspensions of *Anabaena* ATCC 29413 (a gift of Dr. C.P. Wolk) in modified medium D of Kratz and Myers [20], containing 1 mM Na_2CO_3, 10 mM $NaHCO_3$ and 0.15 M NaCl instead of the 17.7 mM $NaNO_3$ (thus raising the ionic strength of the medium by about 370%), were grown under nitrogen-fixing conditions, in continuous turbidostat cultures (New Brunswick Bioflo fermenter, model 30) at 35–36°C, 100 W/m^2 warm white fluorescent light (measured with a YSI radiometer, type 65, at the surface of the growth vessel) and a constant pH of 8.2 ± 0.1, continuously adjusted by feedback-controlled CO_2 supply [21]. The density of the growing cell suspension was kept constant at 3–5 µl packed cells/ml by electronically regulating the rate of fresh medium supply and overflow in response to the apparent optical absorption at 500 nm of a 0.5 cm layer of the growing cell suspension. 6–8 l of the cell suspension were continuously harvested in large bottles thermostatted at 35°C, illuminated with 20–25 W/m^2 incandescent light and bubbled with 1.5% (v/v) of CO_2 in sterile air for at most 1 week. Heterocyst frequency of the harvested filaments was 15–20%, which is about twice the value seen with filaments grown in the absence of 0.15 M NaCl (results not shown).

Filaments were harvested by centrifugation at room temperature, washed twice with sterile 30 mM Hepes-Pipes-KOH buffer and 10 mM EDTA (pH 7.2), finally suspended in 200 ml 10 mM Hepes-KOH, 10 mM $MgCl_2$, 300 mM mannitol, 0.5% BSA and 0.5% lysozyme, and incubated at 35°C for 1 h (50 µl packed cells/ml). Lysozyme-treated filaments were pelleted, washed twice with the mannitol-containing buffer (lysozyme omitted), resuspended in 30 mM Hepes-Pipes-KOH buffer, 1 mM $MgCl_2$, 1 mM PMSF and 0.0075% deoxyribonuclease I (50 µl packed cells/ml, final pH 7.2) and extruded once through a French-pressure cell at 34.5 MPa. This procedure yielded 100% intact heterocysts and a supernatant of broken vegetative cells as examined by phase-contrast microscopy. 1 mM PMSF was included in all following steps of membrane isolation and purification [8]. Heterocysts and vegetative cell extracts were separated by differential centrifugation [22]. The supernatant of the heterocyst pellet, containing crude membranes from vegetative cells, was immediately processed for membrane separation through discontinuous sucrose density gradient

centrifugation followed by repeated recentrifugation of the two principal membrane fractions (yellow plasma membranes and green thylakoid membranes) on fresh gradients [8,9]. After breakage of heterocysts by French-pressure cell extrusion at 138 MPa the crude heterocyst membranes were separated and purified as described for vegetative cell membranes [8,9]. Purified plasma membrane preparations were devoid of spectroscopically detectable chlorophyll.

Oxidation and reduction of reduced and oxidized horse heart cytochrome *c* (type VI from Sigma) or *Saccharomyces cerevisiae* cytochrome *c* (type VIII from Sigma) was followed by dual-wavelength spectrophotometry (Shimadzu UV-300) at 35 °C using an absorption coefficient of 19.5 per mM per cm at 550–540 nm [9,10]. Optimum and reproducible reaction rates were obtained with 6–10 μM cytochrome *c* (initially more than 95% reduced or oxidized) and 0.5–40 μg membrane protein/ml depending on the specific activities of the respective membranes (cf. Fig. 1). The data given in the figures and tables are mean values from at least five independent preparations, standard deviations ranging within ± 10–15% of the corresponding mean.

Affinity chromatography of OG-solubilized membranes on immobilized yeast cytochrome *c* was performed essentially according to Azzi et al. [23–26] but using OG as the only detergent in all the purification steps [10]. Acid-labile heme groups were extracted from intact heterocysts and vegetative cell extracts, respectively, and quantitized spectrophotometrically as the alkaline pyridine ferrohemochromes [27,28].

SDS-PAGE and immunoblotting of membrane polypeptides were performed according to Refs. 9 and 29. Antibodies used were against the aa_3-type cytochrome oxidases of *Paracoccus denitrificans* (subunits I and II and holoenzyme; cf. Refs. 30 and 31) and rat liver mitochondria (subunit II; Ref. 32). Protein and chlorophyll were determined according to Bradford [33] and Mackinney [34], respectively.

Results and Discussion

Fig. 1 shows spectrophotometric recorder traces of horse heart cytochrome c oxidation and reduction by the membrane preparations used in this study. By far the highest cytochrome oxidase activity was displayed by thylakoid membranes from heterocysts. Yet, the lower activities observed with plasma membranes must also be considered significant since, in view of spectroscopically undetectable chlorophyll in these preparations, they cannot stem from contaminating thylakoid membranes (cf. Refs. 9 and 10). Very low concentrations of KCN were sufficient to block the reaction completely. Other typical inhibitors of mitochondrial cytochrome oxidase were also highly effective with all membranes, while the non-ionic detergent OG provoked a 6-fold stimulation of the activity leaving the characteristic inhibition pattern profile unchanged (Table I). The same mem-

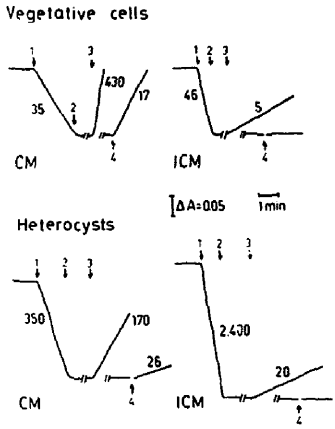

Fig. 1. Oxidation and reduction of horse heart cytochrome *c* by plasma membrane (CM) and thylakoid membrane (ICM) preparations from vegetative cells and heterocysts of *Anabaena* determined by dual-wavelength spectrophotometry. Numbers adjacent to the traces indicate nmol cytochrome *c*/min per mg protein. Arrows indicate addition of (1) 6–10 μM cytochrome *c* (initially more than 95% reduced), (2) 3 μM KCN, (3) and (4) 0.5 mM NADH and NADPH, respectively. Cytochrome oxidase and reductase activities were determined on separate batches of the same sample; 6–10 μM cytochrome *c* (initially fully oxidized) was added to KCN-inhibited membranes 1 min prior to NAD(P)H. Membrane protein concentration (μg/ml) of the samples was 18 and 5 with plasma membranes from vegetative cells (oxidase and reductase assays, respectively), 37 with thylakoid membranes from vegetative cells, 3.5 with plasma membranes from heterocysts, and 0.7 and 5.3 with thylakoid membranes from heterocysts (oxidase and reductase assays, respectively).

TABLE I

RATES OF HORSE HEART FERROCYTOCHROME *c* OXIDATION BY MEMBRANE PREPARATIONS FROM *ANABAENA* VEGETATIVE CELLS AND HETEROCYSTS

Assays were performed using dual-wavelength spectrophotometry at 35 °C. The assay buffer (10 mM potassium phosphate buffer (pH 7.0) total sample volume 0.4 ml) contained membrane protein concentrations as specified for Fig. 1. The reactions were started by addition of concentrated membrane suspensions to cytochrome *c* solution. Except for sulfide, CO and nitrogen, the inhibitors (or activator) were added 1–2 min after the membranes (cf. Fig. 1) Values are given as nmol/min per mg protein). CM_V and CM_H, plasma membranes from vegetative cells and heterocysts, respectively; ICM_V and ICM_H, thylakoid membranes from vegetative cells and heterocysts, respectively. Zero values indicate less than 0.5 nmol cytochrome *c*/min per mg protein.

Additions	Type of membrane preparation			
	CM_V	ICM_V	CM_H	ICM_H
None	35	46	350	2400
1.2 μM KCN	0	0	0	0
5 mM NaN_3	3	5	25	150
0.1 mM Na_2S [a]	14	16	110	550
CO [b]	10	12	95	520
20 mM salicyl aldoxime	4	4	32	190
150 mM NaCl	0	0	0	0
N_2 [c]	0	0	0	0
0.05% *n*-octyl glucoside [d]	230	290	2050	13500

[a] Membranes were incubated with 0.1 mM sulfide for 1 min, centrifuged, washed free of sulfide and resuspended in assay buffer.

[b] Membrane suspensions were flushed with a mixture of $O_2/CO = 1:9$ (v/v) for 5 min prior to the addition of cytochrome *c*.

[c] Assays were conducted in Thunberg cuvettes repeatedly evacuated and flushed with oxygen-free nitrogen.

[d] The response to the inhibitors shown was unchanged in the presence of *n*-octyl glucoside.

branes were able to reduce oxidized cytochrome *c* at the expense of reduced pyridine nucleotides (Fig. 1); however, while NADH was active with all membrane preparations NADPH was accepted by plasma membranes only. This means that plasma membranes and thylakoid membranes are endowed with a specific NADH dehydrogenase, while NADPH dehydrogenase appears to be present in plasma membranes only. Thus, it is rather unlikely that the latter activity could be feigned by ferredoxin:NADP oxidoreductase [35] which is involved as a soluble electron carrier in photosynthetic electron flow, but could not be detected in our thylakoid membrane preparations (cf. Fig. 1). Moreover, having undergone several steps of purification, all of the membranes may be considered 'thoroughly washed' and free of soluble proteins. In contrast to *Anabaena*, neither plasma nor thylakoid membranes from *Anacystis* strictly discriminated between NADH and NADPH [36]. Rather surprisingly, there was no quantitative correlation between the measured rates of electron transport from NAD(P)H dehydrogenase to cytochrome *c* and the rates of cytochrome *c* oxidation (Fig. 1). Reasons for this apparent discrepancy (particularly for the low NADH-cytochrome *c* reductase activity measured with heterocyst thylakoid membranes, which clearly could not account for the high cytochrome *c* oxidase activity) may be selective damage of dehydrogenase or cytochrome oxidase during membrane preparation and/or, more likely, restricted accessibility of the enzymes in the membrane vesicles (in-side-out and/or right-side-out to unknown extents) to exogenous NAD(P)H or cytochrome *c* [37].

The result of affinity chromatography of OG-solubilized *Anabaena* membranes on immobilized yeast cytochrome *c* is shown in Fig. 2. Despite a seemingly clear-cut elution profile, the rather minute amount of partly purified aa_3-type cyto-

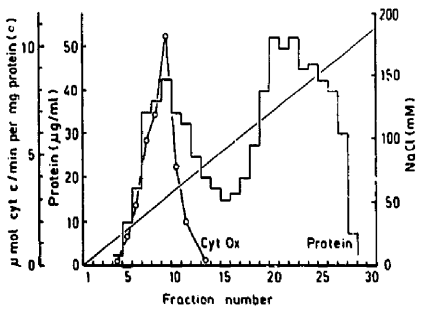

Fig. 2. Elution profiles of protein, and cytochrome oxidase activity, from a column of immobilized yeast cytochrome (cyt) *c* loaded with 1% OG-solubilized heterocyst thylakoid membranes (for details see Materials and Methods). Elution was with a gradient of 0–200 mM NaCl. Reduced minus oxidized difference spectra of fractions 18–25 revealed the presence of *b*-type cytochrome only, while fractions 5–11 mainly contained cytochrome *a* contaminated with cytochrome *b*.

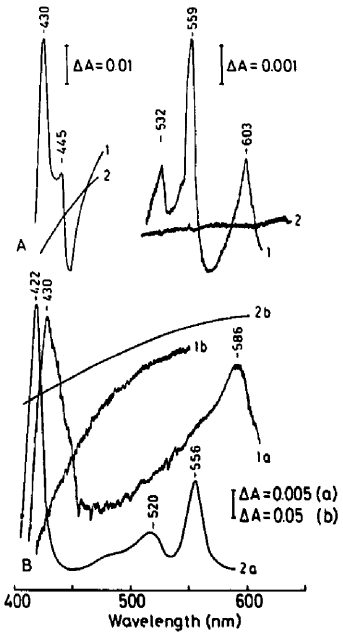

Fig. 3. (A) Dithionite-reduced minus aerated difference spectra of heterocyst thylakoid membranes. 1 mg protein/ml. Trace 2 is the base line (dithionite-reduced minus dithionite-reduced). No qualitative difference was seen between the four different types of membrane used in this study. (B) Dithionite-reduced minus aerated difference spectra of alkaline pyridine hemochromes from heterocysts. 1a, cytohemin (1b, base line), 2a, protoheme (2b, base line). Whole cells (10 mg total membrane protein) were extracted with acidic butanone-2 or acetone, cytohemin and protoheme were separated from each other by celite column chromatography [28], and the cytohemin content was quantitated from the spectra [27]. No difference was observed between extracts from vegetative cells and heterocysts.

chrome oxidase collected from the column was not sufficiently pure to permit unequivocal spectroscopic identification. Generally, the affinity chromatography gave highly variable oxidase binding to the column in different batches. However, reduced minus oxidized difference spectra of OG-solubilized membranes (Fig. 3A) did show the presence of cytochrome aa_3 (α-peak at 603 nm, β- and γ-peaks at 532 and 445 nm less clearly visible) besides, of course, *b*-type cytochrome (α-, β- and γ-peaks at 559, 532 and 430 nm). Acid-labile heme groups were extracted (from whole cells) and characterized by reduced minus oxidized difference spectrophotometry of the alkaline pyridine ferrochemochromes. Besides protoheme (from *b*-type cytochromes), cytohemin (from *a*-type cytochrome) was clearly detected in the spectra (Fig. 3B), thus indicating the presence of aa_3-type cytochrome oxidase. Since, for quantity reasons, whole cells or cell-free extracts, either heterocysts or vegetative cells, were extracted and used as the sources of the cytohemin finally detected, refined localization of the cytochrome on plasma membranes or thylakoid membranes was not possible; yet, the experimental evidence available so far indicates the same type of cytochrome oxidase in all four types of membrane (cf. Fig. 3A and Table I). Quantitative evaluation of the cytohemin spectra (Fig. 3B; Refs. 27 and 28) indicates roughly equal concentrations of cytochrome *a* in both vegetative cells and heterocysts, viz. about 10 ± 3 nmol/mg membrane protein (values much higher than those previously reported, cf. Refs. 17 and 19), despite much higher cytochrome oxidase ac-

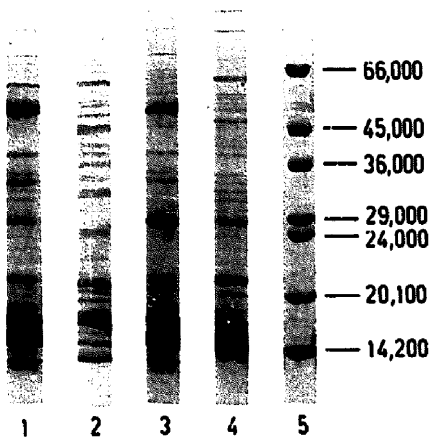

Fig. 4. SDS-PAGE of isolated and purified plasma membranes (lanes 2 and 4) and thylakoid membranes (lanes 1 and 3) from *Anabaena* vegetative cells (lanes 1 and 2) and heterocysts (lanes 3 and 4). Gels were stained with Coomassie brilliant blue. 40 and 80 μg protein was applied to each lane with plasma and thylakoid membranes, respectively. Lane 5 represents the marker proteins (Sigma MW-SDS-70L-Kit).

tivity in heterocysts compared to vegetative cells (cf. Fig. 1 and the following paragraph).

Fig. 4 depicts SDS-PAGE patterns of the different *Anabaena* membranes. It can be seen that each of the membranes is distinguished by a distinct protein inventory corroborating the idea that the membrane preparations were, in fact, different from, and not cross-contaminated with, each other. Transfer of the polypeptides to nitrocellulose by Western blotting, followed by immunological cross reaction with antisera raised against aa_3-type cytochrome oxidase from *P. denitrificans* (subunit I, subunit II and holoenzyme) and rat liver mitochondria (subunit II only) gave a consistent, specific and complementary cross-reaction pattern (Fig. 5) which appears to necessitate the conclusion that the *Anabaena* membranes do contain aa_3-type cytochrome oxidase with (at least) two subunits (of around 48–49 and 36 kDa) showing a considerable degree of amino acid sequence homology to the respective subunits of the oxidases from *P. denitrificans* and rat liver. Moreover, as discussed in the previous paragraph on the grounds of spectral data, the uniform intensity of the cross-reacted bands from different membranes also indicates that, while plasma membranes do indeed contain less cytochrome oxidase than thylakoid membranes, thylakoid membranes from heterocysts do not contain more cytochrome oxidase than those from vegetative cells, despite tremendous higher rates of cytochrome c oxidation displayed by the former (cf. Figs. 1 and 5).

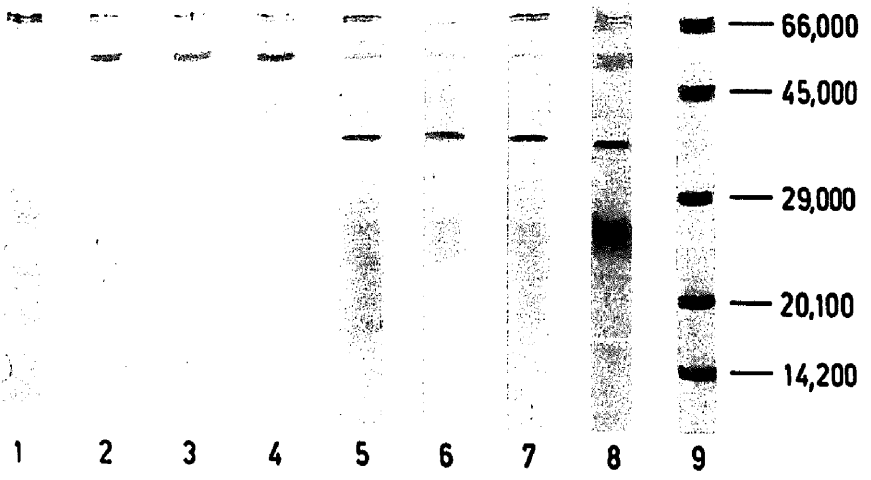

Fig. 5. Immunoblotting of SDS-PAGE-separated polypeptides from *Anabaena* membranes. Lanes 2 and 5, plasma membranes from vegetative cells; lanes 3 and 6, thylakoid membranes from vegetative cells; lanes 4, 7 and 8, thylakoid membranes from heterocysts. Lanes 2–4 and 5–7, cross-reaction with antisera against *P. denitrificans* aa_3-type cytochrome oxidase subunits I and II, respectively. (Cross-reaction with antibody against the holoenzyme consistently gave two bands on each lane corresponding to those seen with antibodies against subunits I and II, separately; not shown, cf. Refs. 8, 9 and 29.) Lane 8, cross-reaction with antiserum against subunit II of rat liver mitochondrial cytochrome oxidase. (The same type of cross-reaction was also obtained with the other membrane preparations; not shown). 40 and 80 μg of membrane protein was applied to each lane with plasma and thylakoid membranes, respectively; no membrane (or other, except antibody) protein was applied to lane 1 showing that the '66 kDa cross-reacting band' (seen on all lanes) probably stems from the artifact induced by mercaptoethanol (or dithiothreitol) present in the sample buffer [41]. The amount of heterocyst plasma membrane material available was insufficient to be tested. Goat anti-rabbit IgG-horseradish peroxidase conjugate was used as the second antibody [29]. Lane 9 represents marker proteins stained with Amido Black 10B after Western blotting.

It may be concluded, therefore, that the specific microenvironment of the cytochrome oxidase in heterocyst thylakoid membranes (e.g., lipid composition, etc.) elicits a much more active state of the enzyme than in thylakoid membranes from vegetative cells. We believe that the very high cytochrome oxidase activity of heterocysts, which is consistent with the high concentration of respirable reserve substances in these specialized cells [4,38], by maintaining low internal oxygen tensions, plays an important role for the so-called respiratory protection of nitrogenase, the highly oxygen-labile enzyme responsible for nitrogen fixation; in addition, high rates of (coupled) respiratory electron flow would correspond with the high ATP requirement (at least 12 ATP/N_2) of this process [39].

Concluding remark

During preparation of this manuscript it was communicated to us that a group at the University of Constance, F.R.G., have succeeded for the first time to isolate and partly purify aa_3-type cytochrome oxidase from *Anabaena* ATCC 29413 heterocysts by the use of fast-performance liquid chromatography [40].

Acknowledgements

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