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Direct Analysis of Phycobilisomal Antenna Proteins and Metabolites in Small Cyanobacterial Populations by Laser Ablation Electrospray Ionization Mass Spectrometry

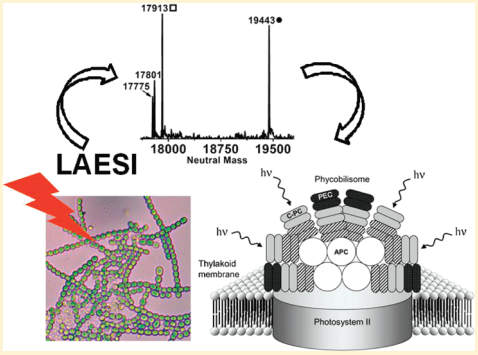
Goetz Parsiegla,[†] Bindesh Shrestha,[§] Frédéric Carrière,[†] and Akos Vertes*[,§]

[†]CNRS, Aix-Marseille Université, Enzymologie Interfaciale et Physiologie de la Lipolyse, UPR 9025, Marseille, France

[§]Department of Chemistry, W. M. Keck Institute for Proteomics Technology and Applications, George Washington University, Washington, D.C. 20052, United States

Supporting Information

ABSTRACT: Due to their significance in energy and environmental and natural product research, as well as their large genetic diversity, rapid in situ analysis of cyanobacteria is of increasing interest. Metabolic profiles and the composition of energy harvesting antenna protein complexes are needed to understand how environmental factors affect the functioning of these microorganisms. Here, we show that laser ablation electrospray ionization (LAESI) mass spectrometry enables the direct analysis of phycobilisomal antenna proteins and report on numerous metabolites from intact cyanobacteria. Small populations ($n < 616 \pm 76$) of vegetative *Anabaena* sp. PCC7120 cyanobacterial cells are analyzed by LAESI mass spectrometry. The spectra reveal the ratio of phycocyanin (C-PC) and allophycocyanin (APC) in the antenna complex, the subunit composition of the phycobiliproteins, and the tentative identity of over 30 metabolites and lipids. Metabolites are tentatively identified by accurate mass measurements, isotope distribution patterns, and literature searches. The rapid simultaneous analysis of abundant proteins and diverse metabolites enables the evaluation of the environmental response and metabolic adaptation of cyanobacteria and other microorganisms.



Due to their significance in energy and environmental and natural product research, as well as their large genetic diversity, rapid analysis of cyanobacteria is being developed using mass spectrometry (MS). Matrix-assisted laser desorption ionization (MALDI) and electrospray MS have been applied for the molecular analysis of intact bacteria and viruses.[1−4] For natural product exploration, the spatial distribution of secondary metabolites has been mapped in a single cyanobacterium *Lyngbya majuscula JHB* filament by MALDI imaging.[5] Ambient mass spectrometry techniques such as desorption electrospray ionization (DESI) MS have been utilized to directly profile lipids in other bacterial samples without extraction.[6] Cyanobacteria, including *Anabaena*, analyzed by MALDI-MS showed strain-specific secondary metabolites that could be used to assess the toxicity of algal blooms.[7] Most of the ambient ionization studies, however, did not report on the protein components of these samples. As laser ablation electrospray ionization (LAESI) MS has been employed to directly analyze the metabolites and lipids in virally transformed T lymphocytes[8] and in single plant cells,[9,10] this method is a promising candidate to rapidly explore small cyanobacterial cell populations. In comparison, LC-ESI-MS would require the extraction of proteins and a lengthy step of chromatography. Among other applications, rapid LAESI-MS analysis would benefit the screening of mutant libraries. It would also be useful for the monitoring of metabolites and phycobilisome composition of cyanobacteria during growth both on a laboratory scale and in a production setting.

Cyanobacteria are gram-negative prokaryotic organisms that possess a cell envelope consisting of three to four layers. Anabaena vegetative cells are barrel shaped and typically 5−7 μm long and larger than 4 μm in diameter.[11] The commonly observed outer membrane is composed of a peptidoglycan layer, which is normally thicker and more cross-linked than in other gram-negative bacteria, and of a cytoplasmic membrane.[12] These outer layers are followed by successive intracellular thylakoid membranes hosting the photosystem and light harvesting complexes of the bacteria, responsible for their energy uptake. In filamentous cyanobacteria, the outer membranes seem to spread continuously around the related bacterial cells of the fiber (see the inset in Figure 1 showing the cells in a filament of *Anabaena* sp. PCC7120) creating a periplasmic space. The periplasmic space is then usable for internal metabolic transport and cell signaling.[13] Under nitrogen deficient conditions, approximately every ninth to fifteenth cell in the filaments loses its ability to perform photosynthesis and differentiate into a specialized heterocyst

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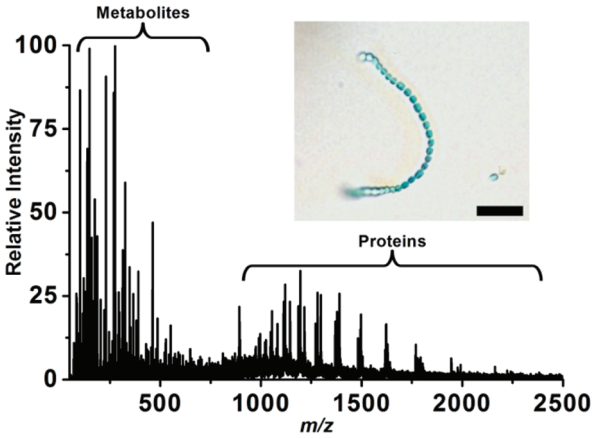

Figure 1. Typical mass spectrum of cyanobacteria *Anabaena* sp. PCC7120 obtained by LAESI-MS analysis of a small colony of cells ($n < 616 \pm 76$). The image in the inset shows a typical cyanobacterial filament from *Anabaena* sp. PCC7120 after rehydration and prior to LAESI-MS analysis. The scale bar represents 20 μm.

cell, which fixes N_2 and supplies the vegetative cells in the filament with soluble nitrogen compounds. Heterocysts are slightly larger than vegetative cells and produce additional layers of cell walls to protect them from oxygen diffusion.

The light harvesting system of cyanobacteria is composed of an intrinsic protein complex of the thylakoid membrane, the photosystems I and II, and extrinsic light harvesting pigment–protein complexes called phycobilisomes.[14] The latter complexes are composed of about 85% phycobiliproteins hosting the chromophores and 15% of unpigmented proteins involved in the phycobilisome core assembly. They are composed of several antenna rods and a base, which transfers the energy to the photosystem II (Figure 2). The light harvesting antenna

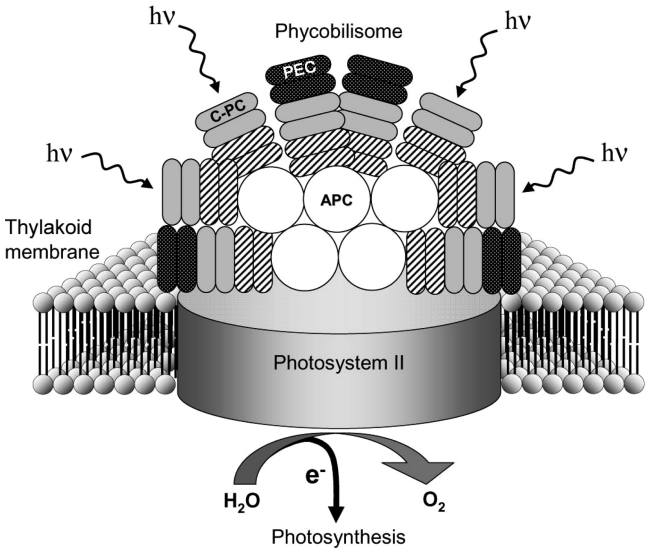

Figure 2. Schematic representation of the phycobilisome protein assembly in *Anabaena* sp. PCC7120, potentially involving phycobiliproteins PEC, C-PC, and APC. Copyright 1996 John Wiley & Sons, Inc. Adapted from ref 24 by permission of John Wiley & Sons, Inc.

phycobiliproteins are composed of an equal number of α- and β-subunits. They are classified according to their observed light absorption and the nature of the covalently linked chromophores.

Cyanobacteria are prototypic photosynthetic living organisms and a useful model for understanding oxygenic photosynthesis and developing new processes for the conversion of carbon dioxide into organic compounds using the energy from sunlight. Because photosynthesis requires a tight association between proteins, small molecules, and lipids from the thylakoid membrane, it is essential to develop rapid methods to address the chemical composition of the diverse components in the photosynthetic machinery. Environmental stresses, such as temperature or osmolyte changes, have known to induce protein modification in cyanobacteria *Anabaena* sp. strain L-31 and tobacco cell culture *Nicotiana tabacum* L. var. Wisconsin 38.[15,16]

Ambient ionization methods, capable of directly detecting cyanobacterial proteins and metabolites, offer an ideal tool for their characterization with minimal perturbation, as up to the moment of analysis, the cells function in their natural environment. In this Letter, we report on the utility of LAESI-MS for the rapid analysis of small cyanobacterial populations. Apart from low molecular weight primary and secondary metabolites and lipids, antenna proteins from the cyanobacterial phycobilisome can be directly identified from intact cells.

■ EXPERIMENTAL SECTION

The cyanobacteria *Anabaena* sp. PCC7120 was a gift from Professor Etienne Weiss. The culture was grown in an in-house assembled 12 L bubble column photobioreactor using 2 L Air/min under pH and temperature static conditions (7.6 and 25 °C, respectively) and permanent artificial light irradiation (fluorescent tube, daylight, 3700 Lux/m^2) (see Table S1a in the Supporting Information). The cyanobacteria were harvested in their exponential growth phase and were lyophilized from 0.7 g wet material from a 14-day culture containing 700 mg/L dry mass. *Anabaena* sp. PCC7120 are known to undergo dehydration and rehydration in their natural environment and remain viable.[17] After rehydration and before the LAESI experiments, the integrity of the cells was checked by microscopy (see the inset in Figure 1). Lyophilization was used for ease of cell storage and transportation. The number of analyzed cells was derived from counting the average number of cells present in an area corresponding to the size of the ablated spot size. In this area, the ablated cells belonged to several filaments. At typical cell densities, $n < 616 \pm 76$ vegetative *Anabaena* cells were present in the 300 μm focal spot. They were ablated by 2.94 μm wavelength laser radiation at 20 Hz repetition rate with 5 ns pulse width produced by an optical parametric oscillator pumped by a Nd:YAG laser (Opotek, Carlsbad, CA, USA) (see Table S1b in Supporting Information). The material ejected by the ablation was intercepted by an electrospray plume produced by applying ~3200 V to a stainless steel emitter (id:50 μm, New Objective, Woburn, MA, USA) delivering 50% methanol solution acidified with 0.1% acetic acid (v/v). Positive ions produced by LAESI were sampled by a mass spectrometer with its orifice on the same axis as the electrospray emitter placed 10–12 mm away from the emitter tip. The sample was placed on a precleaned glass microscope slide. The quadrupole time-of-flight mass spectrometer (Q-TOF Premier, Waters Co., Milford, MA) afforded a mass resolution of $m/\Delta m > 6000$ (fwhm). Whereas the absolute peak intensities fluctuate up to 20% between laser shots, the variations in the intensity ratios in the averaged spectra are below 10%. Multiply charged protein ion peaks were

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deconvoluted by the maximum entropy method (MaxEnt 1, Waters Co., Milford, MA).[18] The CyanoBase genome database at the URL http://genome.kazusa.or.jp/cyanobase/Anabaena was used to obtain protein sequences.

RESULTS AND DISCUSSION

A typical mass spectrum of a rehydrated colony of cyanobacteria obtained from LAESI-MS analysis is shown in Figure 1. Numerous metabolite and lipid ions are observed below m/z 900; additionally, robust signals are found for multiply charged protein molecules between m/z 900 and 2500. The latter are in the +8 to +18 charge states (see Figure 3) and correspond to

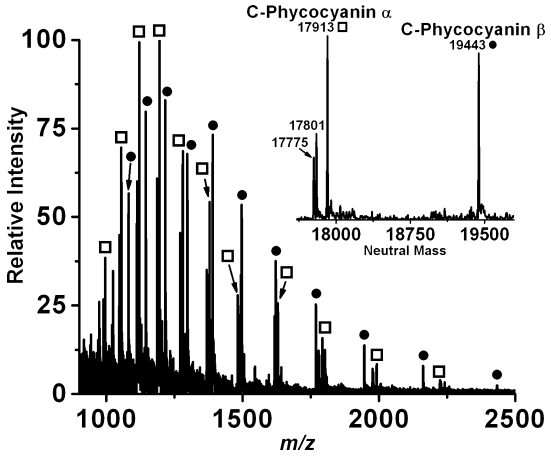

Figure 3. LAESI mass spectrum of cyanobacteria *Anabaena* sp. PCC7120 between m/z 900 and 2500 showing multiply charged protein ions. Multiply charged ions in the +8 to +18 charge states annotated with the open squares (□) and solid circles (●) correspond to two proteins with molecular weights of 17 913 Da and 19 443 Da, respectively. The deconvoluted spectrum is shown in the inset. Two other protein peaks of 17 775 Da and 17 801 Da are also observed in the deconvoluted spectrum.

two main proteins with molecular masses of 17 913 Da and 19 443 Da, respectively (see the inset in Figure 3). The deconvoluted spectrum also reveals the presence of two other peaks corresponding to proteins of approximately 17 775 Da and 17 801 Da (see the inset in Figure 3). These four protein peaks were grouped in two pairs of similar intensity.

We searched the CyanoBase[19] annotated genome database for the studied species to obtain protein sequences which might correspond to the observed masses. Taking into account their significant contribution to the cell dry weight of cyanobacteria during the exponential growth phase[20] and their location in the thylakoid membranes close to the surface, we focused our attention on the family of phycobilisome proteins. The *Anabaena* genome sequence contains ORFs for three types of phycobiliproteins: phycoerythrocyanin (PEC), phycocyanin (C-PC), and allophycocyanin (APC). Adjusted calculated masses for these proteins were obtained by adding two types of post-translational modifications to their primary structures: pigments and N-methylations. In the first case, we used the mass of the pigment phycocyanobilin ($C_{33}H_{38}N_4O_6$ with calculated M_w = 586.678 Da) as measured by Fu et al.,[21] which is covalently linked to some of the cysteines via a nucleophilic addition reaction. Two copies of this pigment or its isomers are linked to the β-subunits of the PEC or C-PC while all other subunits only contain one pigment.[14] As it has been reported recently, N-methylation occurs at a characteristic sequence motif found only in the β-subunit of phycobiliproteins, while the same motif in α-subunits is not N-methylated, due to their location in the whole protein assembly.[22,23] Table S2 of the Supporting Information summarizes all the adjusted masses including the covalent modifications that could potentially match the masses observed by LAESI-MS. The masses of mature phycobiliproteins are all found in the 17−20 kDa range, while core proteins of the phycobilisome assembly are located above or below this range, as shown in Table S2, Supporting Information. The pair of proteins with masses of 17 913 Da and 19 443 Da determined by LAESI-MS are consistent with the C-PC α-subunit (CpcA; 17 913.02 Da) and the C-PC β-subunit (CpcB; 19 443.01 Da), respectively, whereas the other pair with masses of 17 801 Da and 17 775 Da are consistent with the APC α-subunit (ApcA; 17 801.17 Da) and the APC β-subunit (ApcB; 17 774.26 Da), respectively.

Inspection of the inset of Figure 3 reveals that the intensities of the peaks corresponding to the α- and β-subunits of both detected phycobiliproteins is 1.1:1, i.e., CpcA/CpcB ≈ 1.1:1 and ApcA/ApcB ≈ 1.4:1. This observation is consistent with the generally accepted models for subunit composition of these phycobiliproteins, where Apc and Cpc are assembled in disk shaped subunits composed of trimeric (m = 3) or hexameric (m = 6) $(\alpha\beta)_m$ pairs, respectively.[24−26] Our observation indicates that the direct LAESI mass spectrometric method is able to determine the nature of the subunits and the subunit composition of phycobiliproteins in cyanobacteria.

Comparison of the peak intensities in the inset of Figure 3 for the α- and β-subunits of the two detected proteins, C-PC and APC, in the phycobilisome complex, enables us to estimate their relative abundance in phycobilisomes. Although the ionization efficiencies of the involved phycobiliproteins could, indeed, differ, considering the significant structural homology between them, large differences in their ionization efficiencies are not expected. The peak intensities corresponding to the C-PC α- and β-subunits are approximately twice the corresponding intensities for APC, i.e., indicating a PC/APC ≈ 2.4:1 ratio in the phycobilisome. These results correlate well with the accepted model of *Anabaena* sp. PCC 7120 phycobilisome observations in "high light" chromatic adaptation,[24] indicating that direct LAESI mass spectrometry of intact cyanobacteria can be used to explore the chromatic adaptation of their phycobilisomes.

After the background ion intensities in the mass range between m/z 50 and 500 were subtracted, more than 120 ionic species were attributed to the *Anabaena* cells. Approximately 30 of them were putatively assigned to small metabolites and lipids. Some of these tentative assignments included amino acids (alanine, arginine, aspartic acid, tyrosine, and valine), sugars (e.g., hexose), and lipids (e.g., monoacylglyceride, MG(16:0)). Nucleotides involved in nucleic acid and energy metabolism, such as adenosine monophosphate (AMP) and deoxyadenosine monophosphate (dAMP), were also found. The tentative ion assignments are listed in Table S3 of the Supporting Information.

In the genome of *Anabaena* sp. PCC7120, the ORF of three phycobiliproteins, namely, C-PC, C-PEC, and C-APC, can be found, which have different amino acid sequences and absorption spectra but all may contain a phycocyanobilin chromophore (see Table S2 in Supporting Information). In the cylindrical light harvesting antennae of the phycobilisome, multimeric assemblies of each of these light harvesting proteins

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are arranged in a vertical cascade to channel the excitation energy from the highest to the lowest level. This implies that the PEC subunits are located at the top of the rod followed by C-PC subunits, which sit on an APC core (see Figure 2). The architecture of the light harvesting antenna may vary in composition and length from species to species and as a function of growth conditions.[27] The short data acquisition time for the LAESI method described in this article can be the basis of a high throughput approach to study the phycobiliprotein composition of the light harvesting antennae in different species and under different environmental conditions.

C-phycocyanin was reported to be one of the major protein components in the cyanobacteria *Anabaena* and *Spirulina* during the exponential growth phase, representing as much as 12−20% of their whole cell dry weights.[28] Our findings suggest that C-PC and APC constitute the majority of the phycobiliproteins in *Anabaena* sp. 7120 under the applied growth conditions. No ions corresponding to PEC could be found even though published data on the biochemical analysis of *Anabaena* sp. 7120 phycobilisomes had reflected the presence of all three phycobiliproteins.[24] Our findings are consistent with recent studies using fluorescence and absorption spectra of whole cells from *Anabaena* sp. 7120, in which only signals of APC and C-PC but not of PEC were observed.[29] The modulation of PEC presence in the phycobilisomes correlates well with early studies on another cyanobacterium, *Pseudoanabaena* 7409, in which the spectral composition of illuminating light during growth or the nature of the nitrogen source influenced the composition of the terminal phycobilisome antenna proteins.[30−32] For example, phycobilisomes of *Pseudoanabaena* 7409 cultures grown in red light resulted in phycobilisomes with only C-PC while, using green light illumination, C-PC and PE were detected.[33] Studies on the closely related cyanobacteria *Mastigocladus latninosus* showed that the PEC content also might vary with light intensity during cell growth, favoring the presence of PEC at lower light.[34,35] In this latter study, a constant ratio of C-PC to APC of 2:1 was observed independent of the variable amount of PEC subunits. Interestingly, we observe a similar 2.4:1 intensity ratio for C-PC and APC in our measured spectra. On the basis of our data, one can conclude that light conditions during cell growth suppressed PEC integration into the antenna termini.

On the basis of our results, LAESI-MS appears to be a suitable technique for rapid identification of abundant proteins from small populations of intact vegetative cyanobacterial cells. We have shown that LAESI mass spectrometry can provide relative quantitation for the C-PC and APC content of the phycobilisomes, as well as reveal the ratio of the α- and β-subunits within the C-PC and APC phycobiliproteins.

CONCLUSION

We have demonstrated the feasibility of simultaneously analyzing metabolites, lipids, and proteins from cyanobacterial colonies with minimal sample preparation at ambient conditions. The LAESI-MS analysis of small populations between 500 and 1000 cells yields information rich spectra. Protein identification is based on the deconvolution of multiply charged ion peaks followed by mass comparison with proteins obtained from an annotated genome database. Further verification of the putatively identified proteins can be performed by classical MS-based proteomics methods. Comprehensive analysis using multiple methods, such as separation techniques, NMR, FT-IR, etc., is needed for the unambiguous identification of the metabolites.

The diversity of ions detected by the direct analysis of cyanobacteria using LAESI-MS is indicative of the potential of this method for the characterization of other microorganisms. Direct sampling and ionization of small bacterial colonies can be applied in environmental, biofuel, and natural product research, as well as for studying energy harvesting processes in bacteria.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: +1 (202) 994-2717. Fax: +1 (202) 994-5873. E-mail: vertes@gwu.edu. Homepage: http://www.gwu.edu/~vertes.

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Supporting Information

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Goetz Parsiegla[¶†], *Bindesh Shrestha*[¶§], *Frédéric Carrière*[†] *and Akos Vertes*[§*]

[¶] *these authors contributed equally to this work*

[†]CNRS, Aix-Marseille Université, Enzymologie Interfaciale et Physiologie de la Lipolyse, UPR 9025, Marseille, France

[§]Department of Chemistry, W. M. Keck Institute for Proteomics Technology and Applications, George Washington University, Washington, DC 20052, USA

* **Corresponding author.** Department of Chemistry, W. M. Keck Institute for Proteomics Technology and Applications, George Washington University, Washington, DC 20052, USA
Phone: +1 (202) 994-2717; Fax: +1 (202) 994-5873; E-mail: vertes@gwu.edu; Homepage: http://www.gwu.edu/~vertes

Table S1a. Experimental parameters for bacterial growth.

Table S1b. Experimental parameters for laser ablation electrospray ionization (LAESI) mass spectrometry.

Table S2. Calculated masses of phycobiliproteins, phycobilisome core proteins and their relevant derivatives (highlighted species shown in bold are assigned in the spectra in Figure 3).

Table S3. Putative assignments of selected metabolites and lipids between m/z 50 to 500 in a positive ion LAESI mass spectrum of *Anabaena* sp. PCC7120.

Table S1a. Experimental parameters for bacterial growth.

Cyanobacterium species	*Anabaena* sp. PCC7120
Growth condition	12 L bubble column photo-bioreactor using 2 L Air/min
Growth pH	7.6
Growth temperature	25°C
Growth lighting	fluorescent tube, daylight, 3700 Lux/m^2 or 49.95 µmol m^{-2} s^{-1}
Length of culture/concentration	14-day culture containing 700 mg/L dry mass
Cyanobacteria sample preparation	Lyophilized from 0.7 g wet material, before LAESI-MS analysis sample was rehydrated in deionized water

Table S1b. Experimental parameters for laser ablation electrospray ionization (LAESI) mass spectrometry.

Electrospray emitter	Stainless steel; i.d., 50 µm (New Objective, Woburn, MA, USA)
Electrospray voltage	+3200 V
Solution supply rate	300 nL/min
Solvent composition	50% (v/v) aqueous methanol with 0.1% (v/v) acetic acid
Laser	Opolette 100, Opotek, Carlsbad, CA, USA
Laser wavelength and pulse width	2.94 µm and 5ns
Laser pulse repetition rate	20 Hz
Ablated spot diameter	300 µm
Laser pulse energy at target	260.5±10.3 µJ
Mass spectrometer	Q-Tof Premier, quadrupole, orthogonal acceleration time-of-flight tandem mass spectrometer, Waters, Milford, MA , USA
MS orifice temperature	80 °C
MS acquisition mode	Positive ions
MS orifice-to-emitter tip distance	10-12 mm
MS orifice axis-to-sample surface distance	10-12 mm
Number of *Anabaena* cells in laser spot	n < 616±76 vegetative cells
Sample plate	Pre-cleaned glass microscope slide
Number of scans combined for a mass spectrum	10 scans corresponding to 200 laser pulses

Table S2. Calculated masses of phycobiliproteins, phycobilisome core proteins and their relevant derivatives (highlighted species shown in bold are assigned in the spectra in Figure 3).

Phycobiliprotein name	Subunit	Number of residues	Calculated protein mass (Da) [a]	Linked pigments [b]	N-methylation sites [c]	Calculated total mass (Da)
Phycoerythrocyanin (PecA)	α	162	17,323.34	1	0	17,910.04
Phycoerythrocyanin (PecB)	β	172	18,152.45	2	0	19,325.85
Allophycocyanin (ApcA)	**α**	**161**	**17,214.47**	**1**	**0**	**17,801.17**
Allophycocyanin (ApcA2)	α	161	17,813.39	1	0	18,400.09
Allophycocyanin (ApcB)	**β**	**162**	**17,173.56**	**1**	**1**	**17,774.26**
Allophycocyanin (ApcD)	α	161	17,680.30	1	0	18,267.0
C-Phycocyanin (CpcA)	**α**	**163**	**17,326.32**	**1**	**0**	**17,913.02**
C-Phycocyanin (CpcB)	**β**	**173**	**18,255.61**	**2**	**1**	**19,443.01**
Phycobilisome core component (ApcF)		168	18,504.86	0	0	18,504.86
C-phycocyanin-associated rod linker protein (CpcC)		287	32,077.92	0	0	32,077.92
Phycoerythrocyanin-associated rod linker protein (PecC)		277	31,164.19	0	0	31,164.19
Phycobilisome core-membrane linker protein (ApcE)		1131	126,757.08	0	0	126,757.08
Phycobilisome core linker protein Lc7.8; (ApcC)		67	7,708.90	0	0	7,708.90
Phycobilisome rod-core linker protein (cpcG1)		278	31,802.81	0	0	31,802.81
Phycobilisome rod-core linker protein (cpcG2)		246	28,520.29	0	0	28,520.29
Phycobilisome rod-core linker protein (cpcG3)		236	27,074.53	0	0	27,074.53
Phycobilisome rod-core linker protein (cpcG4)		252	29,191.64	0	0	29,191.64
Phycobilisome degradation protein; (NblA)		64	7,411.30	0	0	7,411.30
Phycobilisome degradation protein (NblA)		60	7,266.30	0	0	7,266.30

[a] The protein mass, Mw_{calc}, was calculated for the whole sequence without the leading Met using the Compute pI/Mw tool on the ExPASy Proteomics Server.
[b] Number of added chromophores with a mass of 586.7 Da corresponding to isomers phycocyanobilin, phycoerythrobilin and/or phycobiliviolin. [1]
[c] It has been proposed that in *Synechococcus* sp. Strain PCC 7002 and *Synechocystis* sp. Strain PCC 6803, a post-translational methylation (+ 14 Da) of the Asn 71(or 72) residue in the PPGN motif is performed by a specialized enzyme after the α/β-multimeric assembly in the β-subunit.

[1] E. Fu, L. Friedman, H. W. Siegelman, *Biochem J* **1979**, *179*, 1-6.

Table S3. Putative assignments of selected ions between m/z 50 to 500 in a typical positive ion LAESI mass spectrum of *Anabaena* sp. PCC7120.

Metabolites	Chemical Formula	Calculated Mass	Accurate Monoisotopic Mass	Error (mDa)
pyrroline	$C_4H_7N+H^+$	70.0657	70.0638	-1.9
alanine	$C_3H_7NO_2+H^+$	90.0555	90.0537	-1.8
cytosine	$C_4H_5N_3O+H^+$	112.0511	112.0491	-2.0
proline	$C_5H_9NO_2+H^+$	116.0712	116.0660	-5.2
valine	$C_5H_{11}NO_2+H^+$	118.0868	118.0821	-4.7
homoserine	$C_4H_9NO_3+H^+$	120.0661	120.0770	10.9
oxoproline or pyrroline hydroxy carboxylate	$C_5H_7NO_3+H^+$	130.0504	130.0472	-3.2
leucine or isoleucine	$C_6H_{13}NO_2+H^+$	132.1025	132.1010	-1.5
adenine	$C_5H_5N_5+H^+$	136.0623	136.0614	-0.9
trigonelline	$C_7H_7NO_2+H^+$	138.0555	138.0516	-3.9
aspartic acid	$C_4H_7NO_4+H^+$	148.0484	148.0572	8.8
acetylserine or glutamic acid	$C_5H_9NO_4+H^+$	148.0610	148.0572	-3.8
guanine or hydroxyadenine	$C_5H_5N_5O+H^+$	152.0572	152.0525	-4.7
sym-homospermidine	$C_8H_{21}N_3+H^+$	160.1814	160.1780	-3.4
quinolinediol	$C_9H_7NO_2+H^+$	162.0555	162.0521	-3.4
phenylalanine	$C_9H_{11}NO_2+H^+$	166.0868	166.0772	-9.6
arginine	$C_6H_{14}N_4O_2+H^+$	175.1195	175.1197	0.2
acetylornithine	$C_7H_{14}N_2O_3+H^+$	175.1083	175.1197	11.4
tyrosine	$C_9H_{11}NO_3+H^+$	182.0817	182.0744	-7.3
glutamyl-putrescine	$C_9H_{19}N_3O_3+H^+$	218.1505	218.1484	2.1
succinyl-homoserine	$C_8H_{12}NO_6+H^+$	219.0743	219.0807	6.4
ergothioneine	$C_9H_{15}N_3O_2S+H^+$	230.0963	230.0940	-2.3
aspartyl-lysine or glutamyl ornithine	$C_{10}H_{19}N_3O_5+H^+$	262.1403	262.1426	2.3
adenosine	$C_{10}H_{13}N_5O_4+H^+$	268.1046	268.1018	-2.8
FA (16:1)	$C_{16}H_{30}O_2+K^+$	293.1883	293.1803	-8.0
FA (18:3)	$C_{18}H_{30}O_2+K^+$	317.1883	317.1803	-8.0
FA (18:2)	$C_{18}H_{32}O_2+K^+$	319.2040	319.2020	-2.0
MG (16:0)	$C_{19}H_{38}O_4+H^+$	331.2849	331.2829	-2.0
deoxyadenosine monophosphate (deoxy-AMP)	$C_{10}H_{14}N_5O_6P+H^+$	332.0760	332.0748	-1.2
adenosine monophosphate (AMP)	$C_{10}H_{14}N_5O_7P+H^+$	348.0709	348.0717	0.8
hexose	$C_{12}H_{22}O_{11}+Na+$	365.1060	365.1062	0.2
hexose	$C_{12}H_{22}O_{11}+K^+$	381.0799	381.0799	0.0
punaglandin 3	$C_{25}H_{33}ClO_8+H^+$	497.1942	497.1948	0.6

[a] The monoisotopic masses were calculated using the NIST Isotope Calculator package (ISOFORM, Version 1.02).
[b] Fatty Acyl (FA) and monoglyceride (MG) species are identified by the total length of the acyl chain(s) and the number of double bonds in parentheses.