Interaction of Phycobilisomes with Photosystem II Dimers and Photosystem I Monomers and Trimers in the Cyanobacterium *Spirulina platensis*†

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ABSTRACT: Distribution of phycobilisomes between photosystem I (PSI) and photosystem II (PSII) complexes in the cyanobacterium *Spirulina platensis* has been studied by analysis of the action spectra of H_2 and O_2 photoevolution and by analysis of the 77 K fluorescence excitation and emission spectra of the photosystems. PSI monomers and trimers were spectrally discriminated in the cell by the unique 760 nm low-temperature fluorescence, emitted by the trimers under reductive conditions. The phycobilisome-specific 625 nm peak was observed in the action spectra of both PSI and PSII, as well as in the 77 K fluorescence excitation spectra for chlorophyll emission at 695 nm (PSII), 730 nm (PSI monomers), and 760 nm (PSI trimers). The contributions of phycobilisomes to the absorption, action, and excitation spectra were derived from the in vivo absorption coefficients of phycobiliproteins and of chlorophyll. Analyzing the sum of PSI and PSII action spectra against the absorption spectrum and estimating the P700:P680 reaction center ratio of 5.7 in *Spirulina*, we calculated that PSII contained only 5% of the total chlorophyll, while PSI carried the greatest part, about 95%. Quantitative analysis of the obtained data showed that about 20% of phycobilisomes in *Spirulina* cells are bound to PSII, while 60% of phycobilisomes transfer the energy to PSI trimers, and the remaining 20% are associated with PSI monomers. A relevant model of organization of phycobilisomes and chlorophyll pigment−protein complexes in *Spirulina* is proposed. It is suggested that phycobilisomes are connected with PSII dimers, PSI trimers, and coupled PSI monomers.

Cyanobacteria are prokaryotes that perform oxygenic photosynthesis (*1*, *2*). The role of the light-harvesting antenna in cyanobacteria is performed by phycobilisomes, extramembraneous supramolecular assemblies of phycobiliproteins (*3*−*5*). Hemidiscoidal phycobilisomes, present in most cyanobacteria, consist of a tricylindrical allophycocyanin (APC)[1] core and a fan of six lateral cylinders made up of C-phycocyanin (CPC) or a combination of CPC with C-phycoerythrin or phycoerythrocyanin. The length of the lateral cylinders varies with growth light conditions and between species (*6*). Pigments of the lateral cylinders transfer the absorbed energy to the APC core, which incorporates two terminal chromophoric energy emitters, dubbed the anchor protein and the α-subunit of allophycocyanin B (*6*, *7*). These components transfer the energy further on to the chlorophyll antenna.

Cyanobacteria are relatively poor in PSII compared with PSI (*1*, *2*, *8*), and phycobilisomes are generally assumed to be employed mainly, if not exclusively, as the external antenna of PSII (*9*, *10*). This was shown early by the first known action spectra of photoreaction II (*11*), by low-temperature fluorescence excitation spectra of PSII antenna chlorophyll (*12*−*14*), and by results of time-resolved fluorescence spectroscopy (*10*, *15*, *16*). Low-temperature fluorescence emission spectra of cyanobacteria measured under excitation in the phycobiliproteins absorption region are dominated by chlorophyll bands at 685 nm (F685) and 695 nm (F695) attributed to PSII (*17*, *18*). Along with the spectral data, in vitro preparations were obtained that consisted of phycobilisomes and fragments of thylakoid membranes capable of oxygen evolution (*5*, *19*).

These experimental data clearly showed the connection of phycobilisomes with PSII. However, it was eventually found out that part of the energy absorbed by phycobilisomes is transferred to PSI. For instance, 77 K emission spectra of chlorophyll measured under excitation of phycobilisomes, in addition to fluorescence peaks of PSII, show distinct, though less intense, bands at 715−730 nm (F730) attributed to PSI, while PSI fluorescence excitation spectra exhibit maxima attributed to phycobiliproteins (*12*, *13*, *17*, *18*). Time-resolved 77 K fluorescence spectra of cyanobacteria measured under excitation of phycobilisomes showed a parallel increase of PSII and PSI chlorophyll emissions along with a decrease of their own fluorescence (*15*, *16*). Transfer of energy from phycobilisomes to PSI in cyanobacteria was also revealed by oxidation of P700 in the light absorbed by phycobilisomes, which is recognized by photobleaching of absorption at 705 nm (*20*) and by the EPR signal from the

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[1] Abbreviations: APC, allophycocyanin; CPC, C-phycocyanin; F685 (F695, F730, F760), fluorescence emission bands at 685 nm (695, 730, 760 nm); PSI (II), photosystem I (II); P700, primary electron donor of PSI; P680, primary electron donor of PSII.

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formed cation radical of P700 (*21*). Efficient energy transfer from phycobilisomes to PSI has been found in cyanobacterial heterocystes that lack PSII (*22*). Phycobilisome bands are clearly visible in action spectra of PSI-dependent reversible photoinhibition of respiration in PSII-deficient mutants of *Synechocystis* (*23*) and in the wild-type cells of cyanobacteria (*24*, *25*).

In PSII-less mutants of cyanobacteria phycobilisomes stay bound in the intact form to the photosynthetic membrane (*23*, *26*). The phycobilisome−thylakoid membrane supercomplex, which contains both PSII and PSI, was reported for cyanobacterium *Spirulina platensis* (*27*). Purified phycobilisome samples incorporate significant amounts of ferredoxin:$NADP^+$ reductase, an extrinsic membrane protein functionally connected to PSI (*28*, *29*). The PSI complex isolated from the cyanobacterium *Chlorogloea fritshii* contains some amounts of APC, the main component of the phycobilisome core (*30*).

Presumably, phycobilisome−PSI supercomplexes are highly labile in vivo (*31*, *32*). The data that demonstrate energy migration from phycobilisomes to PSI do not provide, with a few exceptions (*20*, *24*), quantitative estimations of the proportion of phycobilisomes coupled to each photosystem. For this reason, and probably also because the association of phycobiliproteins with PSII was identified much earlier (*11*), there is no common view on the interaction of phycobilisomes with PSI. The assumption that some of the phycobilisomes transfer energy to PSI obviously requires determination of the stoichiometry between phycobilisomes and complexes of the two photosystems. Besides, it is necessary to elucidate the actual structures of PSI and PSII that are linked to these antenna assemblies. It is known that PSII exists in vivo in the form of dimers (*33*, *34*), and recently it was proved that PSI exists in cyanobacterial photosynthetic membranes in both monomeric and trimeric forms (*35*−*37*). Generally, absorption and fluorescence spectra of PSI monomers and trimers are virtually identical, which makes it rather difficult to estimate their contents in vivo (*38*). In contrast to the majority of cyanobacteria, spectral properties of these PSI complexes in the filamentous cyanobacterium *Spirulina* differ significantly. PSI trimers from *Spirulina* contain the minor long-wavelength antenna chlorophyll with the absorption band at 737 nm and low-temperature fluorescence at 760 nm (F760) (*39*, *40*). The intensity of F760 strongly depends on the redox state of P700: uniform kinetics of P700 oxidation and of F760 decrease showed that the long-wavelength fluorescence is quenched by the cation radical of P700 formed in the light (*41*, *42*). When added dithionite prevents accumulation of $P700^+$, the extremely long-wavelength form of chlorophyll serves as the terminal energy trap that accumulates the energy from the bulk chlorophyll of PSI and intensely emits 77 K fluorescence at 760 nm. The 760 nm emission band is an intrinsic marker that allows to distinguish PSI trimers in isolated membranes and within *Spirulina* cells (*41*, *43*, *44*). The F760 chlorophyll probably originates from the pigment−pigment interaction of PSI monomeric complexes in the course of their trimerization (*44*).

Two independent types of measurements provide immediate evidence to the transfer of energy from phycobilisomes to the photosystems. The first one is measurement of action spectra related to photochemical activity of PSI or PSII. The second one is sensitizing of chlorophyll emission by phycobilisomes observed in low-temperature fluorescence excitation spectra. Phycobiliprotein maxima in both types of spectra indicate the participation of phycobilisomes in the functions of the photosystems. In the present work we have used action spectra and 77 K fluorescence excitation spectra to identify the destinations and the shares of excitation energy migration from phycobilisomes to PSI and PSII in *Spirulina* cells. Besides, the unique fluorescence property of PSI trimers in *Spirulina* enabled us to evaluate the stoichiometry of PSI trimers and monomers and to quantify the ratios between phycobilisomes and these two parts of PSI.

MATERIALS AND METHODS

Culture and Growth Conditions. An axenic culture of the filamentous cyanobacterium *S. platensis* strain P 511 was obtained from the IPPAS Culture Collection of Microalgae, Institute of Plant Physiology, Russian Academy of Sciences. A batch culture was grown in 40 mL of Zarrouk's medium (*45*) in 100 mL flasks at 28 °C under continuous white light (60 μE m^{-2} s^{-1}). Seven-day-old culture was used for experiments.

Absorption, Fluorescence Emission, and Fluorescence Excitation Spectra. Absorption spectra of *Spirulina* cells were measured using a Hitachi 557 spectrophotometer with a slit width of 2 nm. The sample was placed in a 0.1 mm thin layer, and 40% of glycerol was added to the sample to reduce light scattering. To compare the low-temperature absorption spectrum with 77 K fluorescence excitation spectra, the cell suspension was dabbed onto a paper filter, and the sample was frozen in liquid nitrogen. Fluorescence excitation and emission spectra were recorded at 77 K using an improved MPF-4 (Hitachi) spectrofluorometer. Data from the fluorometer were retrieved over a PC interface; emission and excitation spectra were corrected to the spectral sensitivity of the photomultiplier and to the lamp emission spectrum, respectively. In both the excitation and measuring monochromators, the slit width was 4 nm. Before spectral measurements the cells were harvested by centrifugation and resuspended in the growth medium at a chlorophyll concentration of 50 μM. The cell suspension was dabbed on a paper filter of 1 cm diameter and rapidly frozen in liquid nitrogen. The samples had an optical density of 0.1 ± 0.01 in the red absorption maximum of chlorophyll, and reabsorption of emitted fluorescence was negligible. The fluorescence measurements of the samples were carried out without addition of glycerol, which altered emission and excitation spectra in the phycobiliproteins spectral region in the same way as it has been reported for cells of other cyanobacteria (*20*). To record F760 fluorescence of the long-wavelength antenna chlorophyll in situ, the cells were placed in 200 mM Tris buffer (pH 9.5) containing 0.5% (w/v) of sodium dithionite. The cell suspension was placed on a paper filter and after 1 min of incubation with dithionite cooled to 77 K in bright light (*35*, *44*). Chlorophyll in an acetone solution was used as a standard to determine the fluorescence intensity of the samples.

Photochemical Action Spectra. Polarographic measurements of action spectra of the flash-induced gas-exchange reactions related to photochemical activity of both PSII (O_2 evolution) and PSI (anaerobic H_2 photoproduction) were

carried out as previously described (*25*, *46*). Assays of cells deposited on the surface of a bare platinum electrode in a 50 mM sodium phosphate buffer with 50 mM KCl, pH 6.8, had a chlorophyll equivalent layer density of 1−2 μg cm^{-2}. O_2 evolution upon actinic light was recorded in the presence of a weak background illumination at a wavelength of more than 700 nm. H_2 photoevolution measurements were carried out under anaerobic conditions after 2 h dark adaptation of the cells in the medium equilibrated with pure argon. Action spectra were recorded using intermittent actinic illumination of 1 s light flashes with variable, 20−60 s, dark intervals. The spectral half-width of the monochromatic beam was 1−3 nm, and its intensity was below 0.2 μE cm^{-2} s^{-1}.

Determination of PSI to PSII Reaction Center Ratio and Effective Antenna Sizes. The ratio of PSII and PSI functional reaction centers was determined from the integral yields of PSII-driven O_2 evolution and PSI-mediated photoinhibition of respiratory O_2 uptake (in the presence of 10 μM diurone) measured polarographically upon single-turnover 1.8 μs saturated light flashes as described earlier (*25*). Effective antenna sizes of the photosystems were calculated from optical cross sections for energy-saturation curves under the flash illumination using the in situ values of optical cross sections of 2.68 and 2.30 Å^2 for a single chlorophyll molecule at 675 nm in isolated PSII core and PSI trimer complexes, respectively (*47*).

Using another approach, the content of PSI and PSII was calculated against the total amount of chlorophyll. For PSI measurements, the whole cells and phycobilisome-free thylakoid membranes were resuspended in the growth medium with a chlorophyll concentration of 50 μM. P700 was determined from the maximal absorption difference between ascorbate-reduced and ferricyanide-oxidized samples at 702 nm in the two-wavelength mode (reference wavelength 730 nm) with an extinction coefficient of 70 mM^{-1} cm^{-1} (*48*). PSII content was evaluated from the quantitative binding of [^{14}C]atrazine with the D1-polypeptide of PSII reaction centers. Cells were incubated for 5 min in the dark with different concentrations of labeled atrazine. The amounts of atrazine bound by the cells were estimated by reference to the counts from a series of known dilution of a label. The PSII content of the cells was assumed to be equivalent to the saturating amount of bound atrazine (*49*). Chlorophyll concentration was estimated from the absorption of 80% acetone cell extracts at 665 nm (*50*).

Pigment Stoichiometry Analysis. The contributions of CPC, APC, and chlorophyll to spectral band intensities were determined for three types of spectra, namely, absorption spectra, action spectra, and fluorescence excitation spectra. This was done by calculating the contributions of CPC, APC, and chlorophyll to the intensity of spectral bands of 625, 652, and 678 nm, the absorption peaks of the three pigments in the total spectrum. Specific molar extinction coefficients for ($\alpha\beta$)-protomers of CPC and APC were estimated as an average of the values available in the literature (*3*, *51*).

CPC: $A_{625} = 1.00$, $A_{652} = 0.20$, $A_{678} = 0.00$;

$$\epsilon_{625} = 225\ \text{mM}^{-1}\,\text{cm}^{-1}$$

APC: $A_{625} = 0.43$, $A_{652} = 1.00$, $A_{678} = 0.04$;

$$\epsilon_{652} = 240\ \text{mM}^{-1}\,\text{cm}^{-1}$$

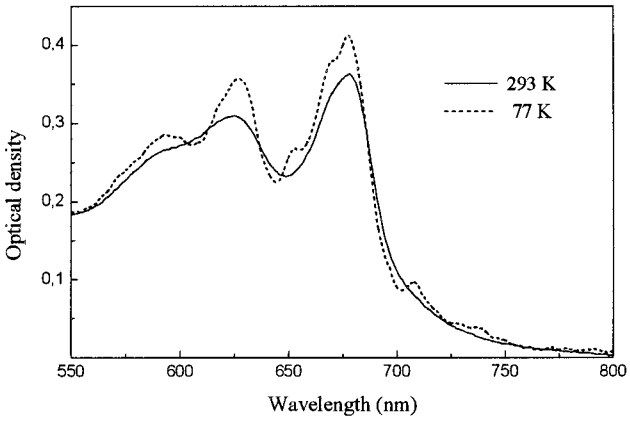

FIGURE 1: Absorption spectra of cell suspension of the cyanobacterium *Spirulina* measured at 293 and 77 K with 40% glycerol.

In vivo chlorophyll (Chl) extinctions were determined for *Spirulina* thylakoid membranes isolated according to the method described earlier (*35*) and carefully washed from phycobilisomes:

Chl: $A_{625} = 0.25$, $A_{652} = 0.24$, $A_{678} = 1.00$;

$$\epsilon_{678} = 64\ \text{mM}^{-1}\,\text{cm}^{-1}$$

Putting these coefficients into a system of linear equations, the contribution of each pigment band in the absorption, action, or fluorescence excitation spectra at the characteristic wavelengths of 625, 652, and 678 nm was defined by the equations:

$$\begin{aligned} A_{625}^{\text{CPC}} &= 1.09A_{625} - 0.46A_{652} - 0.16A_{678} \\ A_{652}^{\text{APC}} &= -0.22A_{625} + 1.10A_{652} - 0.21A_{678} \\ A_{678}^{\text{Chl}} &= 0.01A_{625} - 0.04A_{652} + 0.99A_{678} \end{aligned} \quad (1)$$

A second set of equations was used to determine the molar ratios of CPC, APC, and Chl in the absorption, action, and fluorescence excitation spectra. The following equations were derived from the above ratios adjusted to the molar extinction coefficients ϵ_{625} (CPC), ϵ_{652} (APC), and ϵ_{678} (Chl):

$$\begin{aligned} [\text{CPC}] \times 10^3 &= 4.9A_{625} - 2.1A_{652} - 0.8A_{678} \\ [\text{APC}] \times 10^3 &= -0.9A_{625} + 4.6A_{652} - 0.9A_{678} \\ [\text{Chl}] \times 10^3 &= 0.1A_{625} - 0.7A_{652} + 15.8A_{678} \end{aligned} \quad (2)$$

In fact, the values of [CPC], [APC], and [Chl] are not the genuine molar concentrations in a strict sense. However, their proportions are the actual molar proportions of the pigments. To estimate the ratio of phycobilisomes to chlorophyll, the [APC]:[Chl] ratio was divided by 36 because the core of *Spirulina* hemidiscoidal phycobilisomes contains the amount of chromophores equal to their number in 12 $(\alpha\beta)_3$ trimers of the APC (*3*−*5*). Analysis of the absorption spectra of the same *Spirulina* sample at room and at low temperatures (Figure 1) revealed that eqs 1 and 2 remain valid for the pigment ratio calculations of the 77 K spectra.

To determine the proportions of phycobilisomes and chlorophyll in each photosystem, action spectra of PSI and

Table 1: Ratios of APC/CPC in Phycobilisomes (PBS) and of PBS/Chl Calculated for Various Spectra of *Spirulina*

pigments	absorption spectrum	action spectra		excitation spectra for fluorescence bands		
		PSII	PSI	F695	F730	F760
APC/CPC	1:1.3	1:1.4	1:1.1	nd[a]	1:1.1	1:1.0
PBS/Chl	1:360	1:85	1:380	1:55	1:250	1:370

[a] nd, not determined.

PSII were fitted to the room temperature absorption spectrum of *Spirulina* by means of regression analysis. Likewise, the 77 K excitation spectra were fitted to 77 K absorption spectrum. The regression analysis was performed by the least-squares method using the MS Excel Analysis ToolPak. The fitting procedure for action spectra used about 40 points in the region from 550 to 750 nm with 5 nm spacing. The same procedure for the excitation spectra used more than 350 points in the spectral region from 580 to 740 nm.

RESULTS

The room temperature absorption spectrum of *Spirulina* is typical of cyanobacteria, whose phycobilisomes contain CPC and APC (*7*). The 625 and 652 nm bands of the two phycobiliproteins are best resolved in the spectrum measured at 77 K. CPC shows an additional short-wavelength maximum at 590 nm, which appears as a broad shoulder at room temperature. The 678 nm main chlorophyll band exhibits small 712 and 737 nm peaks of the long-wavelength antenna chlorophyll forms on its red slope (Figure 1). The latter peak belongs to PSI trimers and is characteristic of *Spirulina* thylakoids (*39*, *40*, *43*). Calculation of contributions of the 625, 652, and 678 nm main pigment bands to the total spectrum yielded the CPC to APC to chlorophyll molar ratio of 1.3:1.0:29.0 in the thylakoids. This ratio means that each of the six lateral cylinders of *Spirulina* hemidiscoidal phycobilisomes contains one or two CPC hexamers and that one phycobilisome matches, on average, 360 molecules of chlorophyll. Similarly, the proportions of CPC to APC were determined for other types of spectra, namely, for the action and excitation spectra (Table 1).

The PSII action spectrum for oxygen evolution shows a striking difference from the absorption spectrum. The dominating 625 nm maximum of CPC in the former spectrum indicates an active participation of phycobilisomes in the functioning of this photosystem (Figure 2). The intensity differences of the phycobiliprotein and chlorophyll maxima are so large that the 625 and 652 nm bands overlap the chlorophyll band, observed as just a small shoulder. The action spectrum of PSI, measured separately for H_2 photoevolution, exhibits a 625 nm peak as well, but it is less intense than the 678 nm chlorophyll maximum. Thus, phycobilisomes interact with both PSII and PSI, but in PSI a notably larger fraction of the antenna chlorophyll is bound to these extrinsic complexes. Calculations according to eq 1 for each action spectrum show that in PSII one phycobilisome accounted for 85 chlorophylls, whereas in PSI one phycobilisome corresponds to 380 chlorophyll molecules (Table 1).

The 77 K fluorescence of *Spirulina* cells under 440 nm excitation reveals the F685 and F695 bands of PSII and a more intense F730 band of PSI (Figure 3). These band

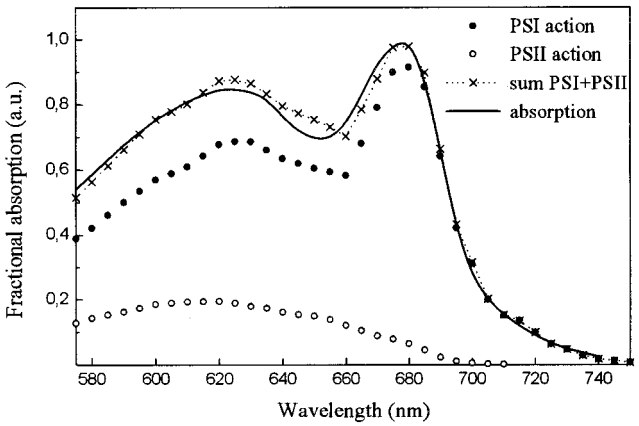

FIGURE 2: Action spectra for photoreactions I and II normalized to add up to the absorption spectrum of *Spirulina* cells. Spectra adjusted by coefficients obtained by regression analysis in MS Excel ($R^2 = 0.99$). Photochemical activities were measured as flash-induced yields of H_2 evolution (PSI) or O_2 evolution (PSII).

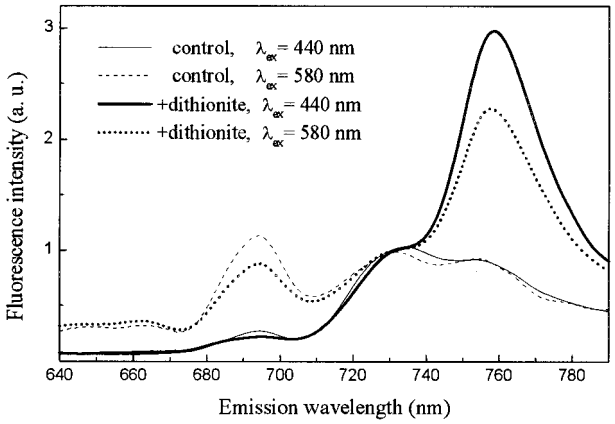

FIGURE 3: 77 K fluorescence emission spectra of the whole *Spirulina* cells. The samples of control or dithionite-incubated cells were excited at 440 and 580 nm; spectra were normalized at 730 nm.

intensities reverse if the emission is excited at 590 nm, the spectral region of phycobilisome absorption. In this case intensities of F685 and F695 increase, and the relative amplitude of the F730 band decreases, indicating effective sensitizing of PSII chlorophyll emission by phycobilisomes. Fluorescence spectra change significantly if the *Spirulina* cells are exposed to reductive conditions by addition of dithionite (Figure 3). An entirely new band of F760 appears, the most long-wavelength and the most intense one (*39*, *43*). Under identical reductive conditions this fluorescence band is characteristic of isolated *Spirulina* PSI trimers which have a respective absorption band at 735−737 nm, as in whole cells (*39*, *44*). The F685, F695, and F730 bands retain their positions in the spectrum in the presence of dithionite. All three bands become less intense against the arising 760 nm band, although F730 does not change its intensity in comparison with the fluorescence of a standard sample (chlorophyll in a solution) (Figure 3). Fluorescence spectra of *Spirulina* samples exposed to reductive conditions, when excited at 590 or 440 nm, preserve the distinctions found without dithionite treatment. If the excitation light is absorbed by phycobilisomes rather than by chlorophyll, then the intensity of the F685 and F695 bands attributed to PSII increases compared with the F730 and F760 bands. Besides, relative intensities of the 685 and 695 nm emission bands

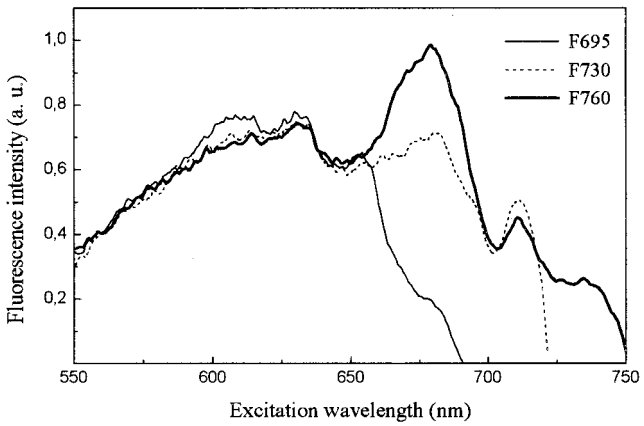

FIGURE 4: 77 K fluorescence excitation spectra of the whole *Spirulina* cells. The spectra of control and dithionite-incubated cells were measured for the emission bands F695, F730, and F760 and normalized at 625 nm.

decrease under reductive conditions (Figure 3). When chlorophyll emission is sensitized by phycobilisomes, the F760 peak appears lower than after direct excitation of chlorophyll at 440 nm. The ratio between PSI emission bands of F730 and F760, derived from the areas of each band less the areas of their own satellite vibration band and the satellite bands of F685 and F695, is also different for excitation at 440 nm (chlorophyll) and 590 nm (phycobilisomes). This ratio amounts to two in the latter case and to three in the former case in favor of the F760 band (Figure 3). Thus, phycobilisomes are bound to chlorophyll that emits at 730 nm in an about 1.5 times bigger proportion than phycobilisomes and chlorophyll fluorescing at 760 nm.

Excitation spectra of the F685−695 bands (PSII) and of F730 band (PSI) in intact cells clearly display the phycobilisome peaks at 625 and 652 nm (Figure 4). This confirms the results of the action spectra analysis (Figure 2), which indicate a connection of phycobilisomes to both PSII and PSI. In the fluorescence excitation spectrum of PSII the 625 and 652 nm peaks are well pronounced, and the shape of the excitation spectrum is similar to the action spectrum of PSII for O_2 evolution. Likewise, the shape of the excitation spectrum for the F730 band is similar to the action spectrum of PSI (Figures 2 and 4). However, the relative intensity of the 625 nm band is higher in the excitation spectrum for F730 compared to both the action spectrum of PSI and to the absorption spectrum. This indicates that the chlorophyll emitting at 730 nm gathers the energy only from a part of the total chlorophyll in PSI and that PSI is likely to have an antenna chlorophyll fraction that does not emit fluorescence. Indeed, as was mentioned above, the additional F760 fluorescence develops after treatment of the cells with dithionite. The intensity of the 625 nm maximum in the excitation spectrum for F760 proved to be relatively lower than that in the absorption spectrum (Figures 1 and 4). The excitation spectrum for the F730 emission band remains unchanged under the reductive conditions.

Altogether, the measurements of fluorescence emission and excitation spectra reveal the presence of two chlorophyll fractions in PSI. The F730 fraction has a smaller chlorophyll to phycobilisome ratio, and its fluorescence intensity is independent from the redox state of P700. The fraction with the F760 emission has a larger ratio of chlorophyll to phycobilisomes, and its fluorescence is observed only for the *Spirulina* cells frozen in the presence of dithionite.

Table 2: Distribution of Chl and PBS between PSI and PSII in *Spirulina*

photosystem	fraction of Chl, %	fraction of PBS, %	reaction center/Chl	effective antenna size, Chl
PSII	5.5,[a] 5.0[b]	20[a,b]	1:800[c]	60[f]
PSI	94.5,[a] 95.0[b]	80[a,b]	1:140,[d] 1:120[e]	97[g]

[a] Based on the fit of the sum of the action spectra to the absorption spectrum. [b] Based on the fit of the sum of the fluorescence excitation spectra to the absorption spectrum. [c] Data obtained using the [^{14}C]atrazine method. [d] P700 content determined in whole cells. [e] P700 content determined in isolated thylakoid membranes. [f] Calculated from light-saturation curves of O_2 evolution upon one-turnover flashes. [g] Calculated from light-saturation curves of H_2 evolution upon one-turnover flashes.

The calculations of the chlorophyll to phycobilisome ratio for the three excitation spectra of the F685−695, F730, and F760 bands, as well as for the absorption spectrum and for the action spectra were performed using eq 2 (Table 1). For the excitation spectrum of the F685−695 bands of PSII the ratio is 55 chlorophyll molecules per phycobilisome. This is smaller than the ratio for the action spectrum of PSII (85 molecules), but both values correspond to the probable connection of phycobilisomes with dimers rather than with monomers of the PSII core complexes. The chlorophyll to phycobilisome ratios in the excitation spectra for the F730 and F760 bands are considerably different from the one yielded by the PSII excitation spectrum. These ratios were estimated to be 250 and 370 chlorophyll molecules per phycobilisome in the excitation spectra of the F730 and F760 bands, respectively (Table 1).

Assuming the whole photosynthetic pigment apparatus of thylakoids is involved in the measured photoreactions, the sum of action spectra of the two photosystems should match the absorption spectrum. An analysis of the absorption spectrum against the sum of action spectra of PSI and PSII proved to be highly accurate. The coefficient of determination (R^2) was 0.99 (Figure 2). This analysis and the results of eq 1 yield two important parameters: the fractions of antenna chlorophyll that belong to each photosystem and the distribution of phycobilisomes between the two photosystems. The proportion of chlorophyll in PSII of *Spirulina* was found to be quite small: the total chlorophyll is distributed between PSII and PSI in shares of about 5% and 95%, respectively. Only 20% of phycobilisomes are bound to PSII, and most phycobilisomes are a constituent of PSI (Table 2). Thus, an average of four phycobilisomes in PSI corresponds to one phycobilisome in PSII.

The excitation energy absorbed by phycobilisomes is transferred to chlorophyll with nearly 100% efficiency (*3*−*5*). Therefore, a total of the three excitation spectra, obtained in the same way as the total of the action spectra, should match the absorption spectrum. The low-temperature absorption spectrum was analyzed against the sum of the three excitation spectra (Figure 5). The analysis proved that the fractions of phycobilisomes and of chlorophyll attributed to PSII are virtually the same as when analyzing the room temperature absorption spectrum against two action spectra (Figure 2), i.e., 20% and 5%, respectively (Table 2). On the other hand, the sum of the two PSI fractions emitting F730

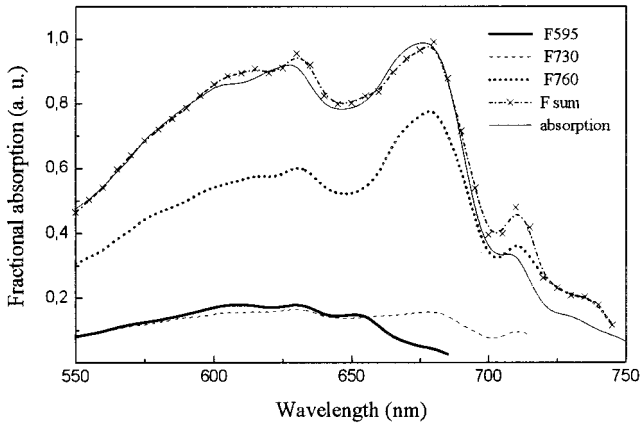

FIGURE 5: 77 K fluorescence excitation spectra of dithionite-incubated *Spirulina* cells for the emission bands F695, F730, and F760. The spectra were normalized so that the sum of the three excitation spectra fits the 77 K absorption spectrum. Spectra adjusted by coefficients obtained by regression analysis in MS Excel ($R^2 = 0.98$).

and F760 accounted for the same amount of pigments as the total fraction of PSI when fitting the action spectra to the absorption spectrum. Chlorophyll and phycobilisomes are allocated between the two fractions of PSI in the following way: 17% of the total chlorophyll and 21% of total phycobilisomes belong to the fraction that emits at 730 nm, and the greater parts of phycobilisomes and chlorophyll, 78% and 59%, respectively, belong to the fraction with the long-wavelength fluorescence F760.

The effective antenna sizes of PSII and PSI photosynthetic units in *Spirulina* were estimated to be 60 and 97 chlorophyll molecules, respectively (Table 2). The first value corresponds better to about two core complexes of PSII, and the last one corresponds to PSI monomers. According to the [^{14}C]atrazine method, there is one P680 reaction center per 800 chlorophyll molecules in the thylakoids of *Spirulina*. According to the "oxidized-minus-reduced" difference absorption spectrum, there is one P700 reaction center per 120 and 140 chlorophyll molecules in the washed thylakoid membranes and in whole cells, respectively. Thus, the ratio of the two photosystems in *Spirulina* is 1:5.7. A very close ratio of PSII to PSI, 1:5.8, was obtained by the polarographic method for the yields of O_2 evolution and PSI-mediated photoinhibition of respiratory O_2 uptake in the cells of *Spirulina* upon saturating light flashes.

The ratios of reaction centers and the fractions of chlorophyll and phycobilisomes in PSII and in the two types of PSI complexes are determined in several ways. A stoichiometric model of the photosynthetic pigment apparatus in *Spirulina* should be consistent with all of these data. According to calculations for the action spectrum and the fluorescence excitation spectrum, one phycobilisome interacts with 55−85 chlorophyll molecules of PSII. This amount best corresponds to the association of phycobilisomes with PSII dimers rather than with monomers. Thus, one phycobilisome−PSII supercomplex incorporates two P680 reaction centers. On the other hand, since the ratio of phycobilisomes in PSII and PSI of *Spirulina* is 1:4 and the ratio of P680: P700 is 1:(5.7−5.8), one phycobilisome associated with two PSII reaction centers is matched by four phycobilisomes bound to about 11 reaction centers of PSI in the photosynthetic membrane. In the trimer fraction of PSI one phycobilisome interacts with 370 chlorophyll molecules. Assuming each phycobilisome is associated with one trimer and dividing this amount by three, we arrive at 123 chlorophyll molecules per P700 in the trimeric fraction of PSI. This value lies within the range reported for PSI monomers (for review, see ref *2*). In the second fraction of PSI, which emits F730, one phycobilisome interacts with 250 molecules of chlorophyll (Table 1). This amount is closest to the antenna size of two PSI monomers. Therefore, phycobilisomes are attached to monomeric and trimeric pigment−protein complexes of PSI, which contain 17% and 78% of the total chlorophyll, respectively. In this case three out of four phycobilisomes in PSI are energetically associated with trimers, and one is connected to coupled PSI monomers. The proposed model of *Spirulina* pigment apparatus based on the energy transfer ratios implies the following relationships of phycobilisomes and photosystems: ~20% of phycobilisomes are linked to PSII dimers, another ~20% are associated with coupled PSI monomers, and ~60% of phycobilisomes are associated with PSI trimers (Figure 6).

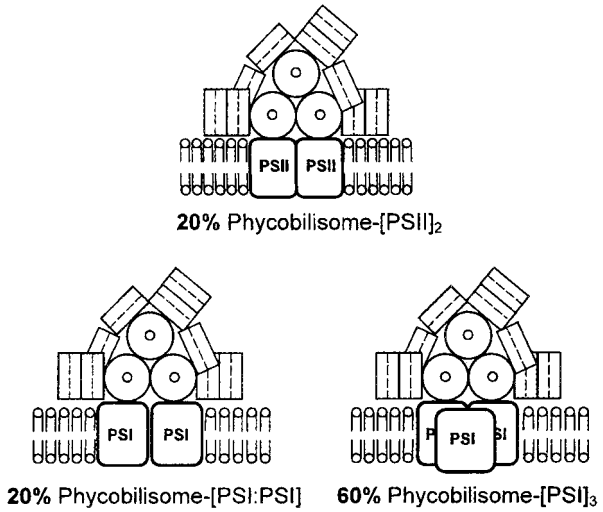

FIGURE 6: Model of interaction of phycobilisomes with the PSII dimers and with the monomeric and trimeric complexes of PSI in thylakoids of the cyanobacterium *Spirulina*. It is suggested that 20% of phycobilisomes are bound to PSII dimers, another 20% are associated to coupled PSI monomers, and 60% are associated to PSI trimers.

DISCUSSION

On the basis of the detailed analysis of action spectra and of fluorescence excitation spectra we obtained a number of parameters that allow to develop a stoichiometric model of the association of phycobilisomes with PSII and PSI complexes in thylakoids of *Spirulina.* We have estimated the fractions of phycobilisomes delivering the light energy to PSI and PSII and the effective antenna sizes of both photosystems, as well as the reaction center ratio and the proportion of CPC and APC within phycobilisomes. The data support the concept that the molecular structure of PSII is made up of dimers and the PSI complex is organized in monomers and trimers, the latter prevailing. Phycobilisomes are coupled to both photosystems, including monomeric and trimeric fractions of PSI. Phycobilisomes detached from pigment−protein complexes of chlorophyll or, vice versa, pigment−protein complexes detached from phycobilisomes are probably negligible transient fractions of the photosyn-

thetic apparatus originating from rapid lateral diffusion of phycobilisomes along the thylakoid surface (*52*).

The PSI:PSII ratio in *Spirulina* was evaluated by two independent methods. First, it was calculated from the ratios of P700 and P680 to the total chlorophyll. Using another approach, the reaction center contents were deduced from the yields of the photochemical gas-exchange reactions specific to each photosystem. Both methods produced very close ratios of 5.7−5.8, whereas a typical value for most cyanobacteria is 2−4 (*9*, *53*, *54*); however, ratios of more than 5 have been reported too (*55*, *56*). The determined PSII and PSI contents correspond to the estimation of 5−6% of the total chlorophyll present in PSII of *Spirulina*. Cyanobacterial PSII contains no more than 5%−10% of the total chlorophyll (*2*), and our estimation of the chlorophyll content of *Spirulina* PSII is in perfect accordance with these data.

The effective antenna size of PSI in *Spirulina* is 97 chlorophyll molecules (Table 2), which is consistent with the size of a PSI monomer (*57*). The effective antenna size of PSII is estimated to be 60 chlorophyll molecules (Table 2). Certain data on the chlorophyll amounts in a monomeric PSII core complex range from as much as 26 to 40 chlorophyll molecules (*2*, *58*). Our value exceeds the chlorophyll contents in a PSII monomer, being closer to the chlorophyll contents in PSII dimers, and implies a dimeric organization of PSII in *Spirulina*. Probably a certain fraction of PSII reaction centers in dimers is closed under our experimental conditions, and the remaining photoactive fraction of the core complexes forms a statistically increased antenna size due to the supposed energy migration from the neighboring inactive core complex.

Phycobilisome bands were found in the action spectra as well as in the low-temperature fluorescence excitation spectra of both photosystems. It gives evidence of energy migration from phycobilisomes to PSII as well as to PSI. Analysis of the absorption spectrum against the sum of PSI and PSII action spectra showed that only 20% of the light energy harvested by phycobilisomes in *Spirulina* is delivered to PSII, while PSI receives the energy from 80% of phycobilisomes. This ratio in favor of PSI is an indication of a direct energy flow from phycobilisomes to PSI in parallel to PSII. Previously, an approach similar to ours was used to fit the absorption spectrum to the sum of two action spectra in the cyanobacterium *Anacystis nidulans*, and the fraction of phycobilisomes interacting with PSI was determined to be 40% (*24*). Although this value is lower than that obtained for PSI in *Spirulina*, it considerably exceeds a value which might indicate a predominant connection of phycobilisomes with PSII complexes. In the case when energy migration from phycobilisomes to PSI was evaluated in a mutant of the cyanobacterium *Synechocystis* 6803 lacking PSII (*20*), the efficiency of 80% was similar to our data. A relatively small part of phycobilisomes connected with PSII correlates with the low contents of this photosystem in pigment apparatus of *Spirulina*.

The fitting of *Spirulina* absorption spectra to the sum of the action spectra of PSII and PSI (Figure 2) and to that of the fluorescence excitation spectra (Figure 5) assumes that all of the phycobiliproteins present in the cell transfer the absorbed energy to antenna chlorophyll. In highly phycoerythrin-rich cyanobacteria some phycobiliproteins are not included in phycobilisomes but serve as reserve protein during nitrogen starvation (*3*, *4*). In this case, the fitting procedure would be incorrect. The phycobilisomes of *Spirulina* do not contain phycoerythrin. A highly sensitive test for the presence of free phycobiliproteins in the cell is their own fluorescence, which should notably exceed the level of chlorophyll emission. The absence of such fluorescence (Figure 3) implies that all phycobiliproteins in *Spirulina* under the experimental conditions were located within phycobilisomes.

The ratio of chlorophyll and phycobilisomes derived from the PSII fluorescence excitation and action spectra along with the data on the PSII effective antenna size shows that *Spirulina* phycobilisomes are most likely to be bound to the dimers of PSII. The surface area of the phycobilisome core connected with the thylakoid membrane has a size similar to that of the PSII dimer (*32*). Each phycobilisome was postulated to be associated by its core with a PSII dimer (*32*−*34*). Our data testify to the model of the phycobilisomes settled on PSII dimers. PSI molecular structures involved in interaction with phycobilisomes have not been considered in earlier analysis of experimental data, although the possible attachment of phycobilisomes to PSI trimers has been proposed (*32*, *59*). This work provides evidence that PSI of *Spirulina* consists of two fractions that have fluorescent characteristics of monomeric and trimeric complexes. Their chlorophyll to phycobilisomes ratios are different. In the major F760 fraction it equals one phycobilisome per one PSI trimer. Because this PSI fraction consists of trimers, the estimated ratio gives perfect grounds for the conclusion that each phycobilisome is bound to one PSI trimer. One phycobilisome in the minor F730 fraction corresponds to two PSI monomers. Therefore, it is possible that each phycobilisome in this fraction is in a contact with one PSI monomer, and half of the monomers are free of phycobilisomes. Alternatively, phycobilisomes could be connected with coupled PSI monomers. We are inclined to accept the second option (Figure 6), because two basal APC cylinders of the phycobilisome core incorporate two terminal energy emitters, and the surface of the phycobilisome core exceeds the cytoplasmic area of one PSI monomer complex (*6*, *32*).

PSI trimers are formed due to the presence of the linker polypeptide PsaL (*60*), but the forces holding possibly coupled PSI monomers together are unknown. The phycobilisome itself may be responsible for stabilizing and coupling the two monomers. Similarly, the mechanism of looser binding of phycobilisomes with PSI, as compared with PSII (*30*, *32*), remains unclear. In contrast to the flat cytoplasmic area of the PSII dimers (*32*, *34*), the surface of the PSI monomers shows major protrusions of three hydrophilic polypeptide subunits, PsaC, PsaD, and PsaE, which extend about 3 nm into the cytoplasm (*57*). These protrusions, which do not cover the whole surface of PSI, would prevent a tight binding of phycobilisomes to PSI complexes (*32*). The amount of PSI core complexes joined in trimers in *Spirulina* was determined to be 4.5 times greater than the amount of the monomer fraction. It is very close to the 4:1 chlorophyll ratio in the trimer and monomer parts of PSI found for *Synechocystis* sp. PCC 6803, according to different fluorescence quantum yields of these two fractions of PSI (*38*). The ratio between PSI trimers and monomers in vivo seems to be variable, although in favor of the trimer fraction, and could be regulated by the presence of the PsaL

polypeptide (*60*). Apparently, regulation of the monomer and trimer contents allows to change the absolute amount of PSI and its ratio to that of PSII in the thylakoid membrane. Earlier it was shown that one-third of the PSI complexes participate in linear electron transport from PSII, and the remaining two-thirds specialize in the cyclic electron flow (*61*, *62*). Therefore, it cannot be excluded that the PSI trimers and monomers play different functional roles in photosynthesis.

PSI monomers incorporate about 2.5 times more chlorophyll compared to monomers of PSII, and a PSI trimer contains about four to five times more chlorophyll than a PSII dimer, which complicates a direct demonstration of energy migration from phycobilisomes to PSI. In the action spectrum (Figure 2) and in the excitation spectrum (Figure 4) of PSII, phycobiliprotein bands are more pronounced than in the spectra of PSI. This gives the wrong impression of the predominant binding of phycobilisomes to PSII. However, as shown by our results, the main part of phycobilisomes in *Spirulina* is connected with PSI, which is consistent with the predominance of PSI in the membranes of cyanobacteria and challenges the idea that phycobilisomes transfer energy mainly, or even exclusively, to PSII.

The photosynthetic apparatus of cyanobacteria can be maintained in two energetically different states, which are defined as state 1, attained after preferential excitation of chlorophyll, and state 2, attained after preferential excitation of phycobilisomes. The state transition is a mechanism of short-term adaptation to unbalanced excitation of PSI and PSII, providing a redistribution of light energy between the two photosystems. The proportion of the energy absorbed by phycobilisomes and delivered to PSI is greater in state 2 than in state 1 (*63*). The state transitions can be achieved also by the control of physiologically relevant factors, such as the redox state of the photosynthetic membrane. Specifically, reductive conditions put the cyanobacterial cell into state 2. Therefore, our measurements of the PSI action spectrum for H_2 photoproduction under anaerobic conditions and fluorescence excitation spectra measurements under dithionite pretreatment correspond to state 2. On the contrary, the conditions of the PSII action spectrum measurements in the presence of background illumination at 700 nm correspond to state 1. Nevertheless, we have performed the fitting of the absorption spectrum of *Spirulina* to the sum of two action spectra, taking into account the fact that this could only reduce the resulting share of phycobilisomes connected with PSI. At the same time, the fitting procedure done for all three excitation spectra measured exclusively in state 2 conditions gave the same phycobilisome ratio in the two photosystems as the action spectra total. Basically, our spectral measurements were performed in state 2 conditions when the energy transfer from the phycobilisomes to PSI is at its highest (*63*). We can make only a preliminary assumption that during the state transitions the content of PSI monomers is not changed, judging by the constant intensity of the fluorescence band at 730 nm after addition of dithionite to the cell. It is an open question how the energy migration from phycobilisomes to PSI and PSII in *Spirulina* would change in state 1. Our methodology does not allow to check this, although other methods, such as time-resolved fluorescence measurements (*16*, *63*), would make this possible.

The molecular mechanism underlying the state transitions in cyanobacteria has not yet been fully understood (*64*). One possible explanation would be a spillover of excitation energy from PSII to PSI. According to this model, direct energy transfer from phycobilisomes to PSI is limited or absent, and the energy migration to PSI is mediated by the antenna chlorophyll of PSII. The spillover assumption would impede quantitative evaluation of the energy migration from phycobilisomes to PSI. The present study shows that most phycobilisomes (about 80%) in *Spirulina* are connected with PSI. In case of spillover the situation should be the opposite; i.e., most phycobilisomes would transfer energy to PSII. Thus, the spillover is an unlikely mechanism of state transitions and of energy migration from phycobilisomes to PSI in *Spirulina*. Most likely, energy is transferred independently to each photosystem.

The obtained ratios of CPC and APC in the *Spirulina* phycobilisomes (Table 1) are in accordance with the data of electron micrographs where one or two hexameric CPC disks could be seen in each lateral phycobilisome rod (*65*). Phycobilisomes present in both parts of PSI contain a smaller amount of CPC than the phycobilisomes bound to PSII (Table 1). Variations in the length of the lateral phycobilisome rods are well-known (*3*, *5*). The different length of CPC phycobilisome rods of *Spirulina* PSII and PSI may be one of the ways to regulate the photosynthetic antenna size in this cyanobacterium. The observed lower ratio of CPC to APC in phycobilisomes belonging to PSI contradicts the model of PSI attachment to the lateral rods of the phycobilisome rather than to its core (*32*) that requires a higher ratio of CPC to APC bands in fluorescence excitation and action spectra.

The ratio of phycobilisomes to the total chlorophyll varies between species of cyanobacteria. The observed change in stoichiometry optimizes photosynthetic efficiency under certain growth and light conditions (*66*, *67*). In the present work we do not consider the dynamics of biosynthesis of phycobilisomes and photosystems or related long-term adaptations of *Spirulina* photosynthetic apparatus. *Spirulina* is a unique cyanobacterium whose PSI trimers are capable of 77 K fluorescence at 760 nm. The extreme long-wavelength emission was also found in other filamentous cyanobacteria: *Pseudoanabaena* sp. (*68*), *Phormidium uncinatum*, and *Nostoc muscorum* (*39*). A further study of PSI and PSII contents and of PSI heterogeneity in these cyanobacteria would allow to compare the stoichiometry of phycobilisomes and PSI and PSII complexes with the data presented above. This investigation is currently in progress.

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