Physiological Significance of Overproduced Carotenoids in Transformants of the Cyanobacterium *Synechococcus* PCC7942

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The functional location of carotenoids in the photosynthetic apparatus of *-crtB* and *-pys* transformants of the cyanobacterium *Synechococcus* PCC7942 was studied and compared with a control strain *-pFP1–3*. These transformants overproduce carotenoids due to the insertion of an additional foreign phytoene synthase gene. A higher carotenoid content was found for *-crtB* and *-pys* transformants both in whole cells and isolated membranes; the *-crtB* transformant was also enriched with chlorophyll. 77-K fluorescence emission and excitation spectra of the phycobilin-free membranes were examined for a possible location of overproduced carotenoids in pigment-protein complexes *in situ*. A similar ratio of the amplitudes of fluorescence bands at 716 and 695 nm emitted by photosystems I and II, found for the three strains, indicates that the stoichiometry between photosystems of the transformants was not changed. Overproduced carotenoids are not located in the core antenna of photosystem I, since 77-K fluorescence excitation spectra for photosystem I of isolated membranes from the studied strains do not differ in the region of carotenoid absorption. When illuminated with light of the same intensity but different quality, absorbed preferentially by either carotenoids, chlorophylls or phycobilins, respectively, oxygen evolution was found always higher in the transformants *-crtB* and *-pys* than in *-pFP1–3* control cells. Identical kinetics of fluorescence induction of all strains under carotenoid excitation did not reveal a higher activity of photosystem II in cells enriched with carotenoids. It is suggested that overproduced carotenoids of the transformants are not involved in photosynthetic light-harvesting; rather they may serve to protect the cells and its membranes against photodestruction.

Introduction

In photosynthetic organisms carotenoids (Cars) are instrumental to protect the pigment-protein complexes against photodestruction caused by high irradiation, and to harvest light by collecting quanta and transferring the absorbed energy to antenna chlorophylls (Chls) (Siefermann-Harms, 1985; Frank and Cogdell, 1996; Young and Frank, 1996). The function of Cars in photosynthetic membranes depends strongly on their location. Cars bound to the reaction center complex mainly protect that center by quenching the triplet state of the special Chl pair. Cars in the core antenna and light-harvesting complexes (LHC) are active both for light-harvesting and protection of antenna by quenching of Chl triplets that prevents the formation of singlet oxygen. Cars accumulated in massive amounts in algae like *Dunaliella* are not located in the thylakoid membranes. They protect cells against photodamage through absorption of the blue region of the spectrum („sunscreen", Ben-Amotz *et al.,* 1989). Recently another function of Cars was found for some pigment-protein complexes: Cars are necessary as a structural component for the biogenesis and stabilization of photosynthetic complexes (see Moskalenko and Karapetyan, 1996 for review). Cars contribute to assembly and stabilization of photosystem II (PSII) complexes (Lehoczki *et al.,* 1982; Humbeck

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et al., 1989; Karapetyan *et al.*, 1991; Markgraf and Oelmüller, 1991) and plant LHCII (Kühlbrandt *et al.*, 1994; Paulsen, 1995), while the reaction center complexes of photosystem I (PSI) may be formed and function without Cars (Lehoczki *et al.*, 1982; Römer *et al.*, 1990; Karapetyan *et al.*, 1991, 1992).

In contrast to higher plants and eukaryotic algae, cyanobacteria lack Chl-containing LHC. Their function is taken over by phycobilisomes containing different phycobilins (PB). Accordingly, Chls in cyanobacteria are bound to the core antenna of the PSI and PSII complexes (Golbeck, 1992). Also cyanobacteria contain high amount of Cars, which are located both in photosynthetic, cytoplasmic and outer membranes as well (Omata and Murata, 1984). The pathway of Car biosynthesis in cyanobacteria is similar to that of eukaryotic photosynthetic organisms, but the Car composition of cyanobacteria and higher plants differs; the core complexes of both photosystems contain mainly β-carotene (Hirschberg and Chamowitz, 1994).

Due to their prokaryotic nature cyanobacteria can be genetically manipulated more easily than higher plants, and thus provide an excellent model system to modify carotenogenesis. The insertion of the phytoene synthase gene *crtB* from either the heterotrophic bacterium *Erwinia uredovora* (Misawa *et al.*, 1990) or from the cyanobacterium *Synechocystis* PCC6803 (the *pys* gene; Martinez-Ferez *et al.*, 1994) into the genome of *Synechococcus* PCC7942 resulted in an increased content of Cars in the transformant strains (Windhövel *et al.*, 1995). It remained unclear, however, where the overproduced Cars are located and what function they exert. Cells of the *-crtB* transformant of *Synechococcus*, enriched in Cars, resulted in an increased maximal photosynthetic capacity, while that of the *-pys* strain, also enriched with Cars, was even lower than the maximum capacity of the control strain *-pFP1–3* (Windhövel *et al.*, 1995). This finding may indicate the binding of overproduced Cars to different membranes in the two transformants.

We tried to determine the location of overproduced Cars in the photosynthetic apparatus by checking their role in light-harvesting in PSI and PSII. Oxygen evolution and the kinetics of fluorescence induction were measured when cyanobacterial transformants were illuminated with blue-green light, absorbed mainly by Cars. The location of overproduced Cars in the antenna of photosystems of the transformants was examined by 77-K fluorescence emission and excitation spectra of PB-free membranes.

Materials and Methods

Bacterial transformants, growth conditions, and preparation of membranes

The following strains, derivatives of strain *Synechococcus* PCC7942-PIM8 (Van der Plas *et al.*, 1990) were examined in this study: *Synechococcus* PCC7942-*pFP1–3* represented the control which includes an ampicillin and a kanamycin resistance gene inserted in its genome. It was compared with *Synechococcus* PCC7942-*crtB* and *Synechococcus* PCC7942-*pys*, containing either the phytoene synthase gene *crtB* or *pys*, respectively (Windhövel *et al.*, 1995), in addition to antibiotic resistance genes and to the endogenous phytoene synthase gene. The cells had been cultivated at 30 °C at 20 μEinstein m^{-2} s^{-1} in mineral medium (BG11) supplemented with methionine, kanamycin and ampicillin (Windhövel *et al.*, 1994), gassed by an air/CO_2 mixture (1.5% CO_2 v/v) until they reached the logarithmic growth phase. For all measurements five independent cultivations were used. The data presented are from a typical experiment; deviations were ± 3%. Membranes were isolated from the cells using French-press disruption (50 Mpa), a double washing step (centrifugation and resuspension in 0.05 M Tris-HCl (tris(hydroxymethyl) aminomethane) buffer, pH 8) was sufficient to obtain PB-free membranes.

Pigment analysis

The Chl and Car content was determined after methanol and petrolether/ether extraction, respectively, and referred to packed cell volume (pcv) measured after centrifugation of 2 ml cell suspension in a graduated microcentrifugation tube. The relative amount of PB (PB/Chl ratio) was calculated from the absorption spectra of cells measured with a Shimadzu UV-300 spectrophotometer. Absorption spectra of isolated membranes were recorded with a Perkin-Elmer Hitachi 340 spectrophotometer.

Photosynthetic activity

Oxygen evolution was measured at 30 °C, using a home-built Clark-type electrode setup (Böger, 1993), with 1.7-ml cell samples (Chl content 5 μg/ml). The cell suspension was illuminated from two sides, total intensity was adjusted to 120 μEinstein m^{-2} s^{-1} which was far from saturating intensity (Windhövel *et al.*, 1995). White light was used to check the function of all pigments. To follow the contribution of various antenna pigments to oxygen evolution, different glass filters (Schott, Mainz, Germany) were used to illuminate cells by spectrally different light (Fig. 1):

1. Red cut-off filter RG-665 providing light of wavelengths longer than 665 nm, absorbed mainly by Chl (red light);
2. Orange cut-off filter OG-570, providing the actinic light of wavelengths longer than 570 nm, absorbed by PB and Chl (orange and red light);
3. Blue-green glass filter BG-18 providing light in the region of 400–600 nm, absorbed mainly by Chl and Car, and partially by PB (blue-green light).

Data of the control strain *Synechococcus* PCC7942-*pFP1–3* was taken as a reference. Due to overlapping absorption bands of the different photosynthetically active pigments it was impossible to use a light quality which is absorbed selectively by one certain pigment only.

Fluorescence induction

The kinetics of fluorescence induction of the cyanobacterial cells were measured at 25 °C using the Hansatech instrument DW3 and a light source with maximum intensity of white light of 660 μEinstein m^{-2} s^{-1}. A special Hansatech blue-green glass filter similar to BG-18, but with higher transmission in the 400–500 nm region, was placed between light source and the optical fiber guide. About 2 ml of the cells in culture medium (Chl content 2.5 μg/ml) were illuminated in the DW3 via an optical guide with 400–500 nm light. Since possible differences in the kinetics of fluorescence transients of transformants could be followed only at low light intensity, the exciting blue-green light was decreased to 30 μEinstein m^{-2} s^{-1} by a neutral filter.

Fluorescence spectra

77-K fluorescence emission and excitation spectra were measured for the PB-free membranes isolated from transformant cells to prevent the overlap of absorption bands of Cars and PB in the region 470–520 nm. Membranes were diluted with 60% glycerol to a final Chl concentration of 0.5 μg/ml, placed in 4 mm quartz tubes and frozen. Spectra of membranes were registered in front-face regime by the spectrofluorometer Fluorolog (Spex Industries, USA) at 77 K (Holzwarth *et al.*, 1993); spectra were corrected for spectral sensitivity of the instrument. Fluorescence emission spectra were measured in the region 650–760 nm with excitation of Chl (at 440 nm) or Car (at 500 nm) and normalized for the main maximum. Fluorescence excitation spectra of PSI in membranes were registered for the emission band at 725 nm.

Results and Discussion

Content of pigments in the transformant cells and membranes

For a general information of the pigment content in transformant strains, the absorption spectra (400–800 nm) of whole cells were recorded (Fig. 1). The absorption maxima at 438 nm (Car+Chl), 625 nm (PB) and 678 nm (Chl) were determined and related to eachother: A438/A678= (Chl+Car)/Chl, A438/A625=(Chl+Car)/PB, and

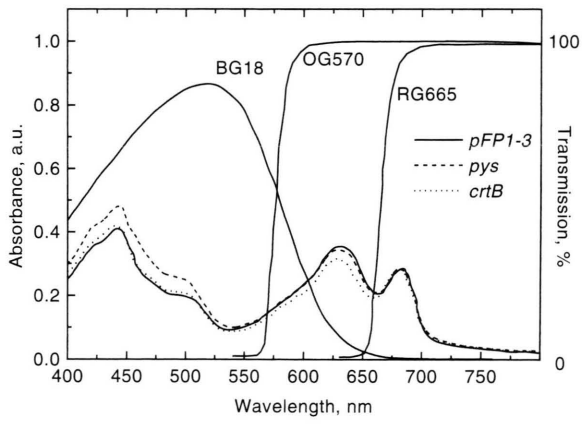

Fig. 1. 293-K absorption spectra of the *-pFP1–3* control strain, *-pys* and *-crtB* transformant cells of *Synechococcus* PCC7942 normalized for 678 nm, and transmission spectra of glass filters BG18, OG570 and RG665 used to measure oxygen evolution by the cells.

A625/A678=PB/Chl (Table I). To check the content of Cars in the membranes isolated from the used strains absorption spectra of membranes were measured (Fig. 2). According to these spectra normalized for the red maximum, membranes of the *-crtB* and *-pys* strains contain more Cars than the membranes from the initial strain (Fig. 2, inset). Strain *-crtB* had more Cars than strain *-pys*, but since transformant *-crtB* was also highly enriched with Chl, the Car/Chl ratio for strain *-pys* was higher than that for *-crtB*. The A438/A678 ratio was found higher for membranes as compared with that of cells since PBs do not contribute to the red absorption peak. (Note that the isolated membranes with A438/A678 ratios (in%) are very similar to those of whole cells of the respective transformant).

Table I. Ratio of pigments (Chl+Car)/Chl=A438/A678, (Chl+Car)/PB=A438/A625 and PB/Chl= A625/A678 calculated from absorption spectra of *Synechococcus* PCC7942 control (*-pFP1–3*) and transformant (*-crtB* and *-pys*) cells, and the ratio (Chl+Car)/Chl=A438/A678 from absorption spectra of isolated membranes (comp. Figs. 1 and 2).

Transformant	Cells			Isolated membranes
	A438/A678	A438/A625	A625/A678	A438/A678
-pFP1–3 (control)	1.53 (100%)	1.24 (100%)	1.24 (100%)	1.90 (100%)
-crtB	1.58 (103%)	1.40 (113%)	1.13 (91%)	1.97 (103%)
-pys	1.73 (113%)	1.37 (110%)	1.27 (102%)	2.08 (110%)

The chlorophyll content was 5 μg/ml; the number in parentheses in all tables represent the percentage of the values referred to control (100%).

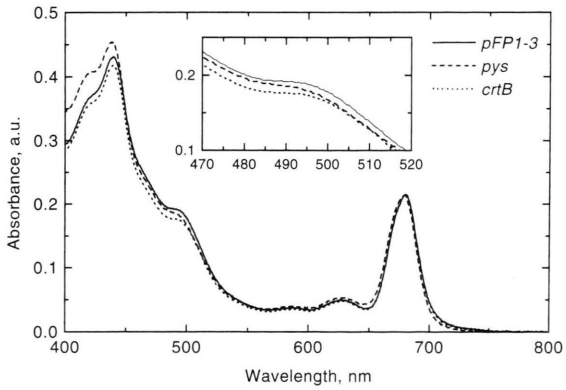

Fig. 2. 293-K absorption spectra of the membranes isolated from *-pFP1–3*, *-pys* and *-crtB* transformant cells of *Synechococcus* PCC 7942 normalized for 678 nm.
Inset: absorption spectra of isolated membranes in the region 470–520 nm.

Table II presents the absolute content of Chls and Cars and their ratio determined after extraction. As compared with the *-pFP1–3* control strain, cells of the two transformants containing an additional phytoene synthase gene were enriched with Car (more than 50%). It should be mentioned that for all transformants the increase of the ratio (Chl+Car)/Chl, calculated from the content of pigments determined after extraction (Table II, last column), was very similar to the increase of the ratio A438/A678 determined from the absorption spectra (Table I). This indicates that the A438/A678 ratio change in % is highly informative for the relative content of pigments. The insertion of foreign phytoene synthase genes obviously also affected the Chl and PB content of the cells (Tables I and II). Based on identical Chl contents, the relative PB content (measured as the ratio A625/A678) of the *-crtB* transformant is lowered by 10% as compared with the *-pFP1–3* and *-pys* strains.

Photosynthetic oxygen evolution

If the overproduced Cars are included in the pigment antenna, one could expect a higher photosynthetic activity of transformant cells (referred to the same Chl content or to pcv) measured by Car excitation with low intensity light. According to the absorption spectra of cyanobacterial cells (Fig. 1), Cars absorb about 20% of the total energy absorbed by all pigments (Chl, PB, Car). The content of Cars in the transformant cells was increased by about 50% (Table II), and their additional light-harvesting effect, calculated to about 10%, might be detected as an increase in photo-

Table II. Content of chlorophyll *a* and colored carotenoids in *Synechococcus* PCC7942 control (*-pFP1–3*) and transformant (*-crtB* and *-pys*) cells grown at a light intensity of 20 μEinstein m^{-2} s^{-1}.

Transformant	Chl	Car	Car/Chl	(Chl+Car)/Chl
	(μg/ml pcv)	(μg/ml pcv)		
-pFP1–3 (control)	5132 (100%)	1049 (100%)	0.204 (100%)	1.20 (100%)
-crtB	6789 (132%)	1621 (155%)	0.239 (118%)	1.24 (103%)
-pys	5408 (105%)	1706 (162%)	0.315 (154%)	1.31 (109%)

synthetic activity of transformant cells under the excitation of Cars. But since probably not all Cars are bound to thylakoids this effect would be less than 10%.

According to Table III (A, B), the photosynthetic activity of *-pys* and *-crtB* cells (compared to that of *-pFP1–3* cells) was found always higher with any light quality tested. This was especially pronounced by relating the data to packed cell volume. Blue-green light is absorbed by Cars, Chl and PB as well. So the increase of photosynthetic activity, possibly due to overproduced Cars, cannot be determined accurately under illumination with blue-green light.

Highest relative photosynthetic activity was obtained in the three strains under illumination with white and orange light, which excite both photosystems simultaneously (Table III, C). With all strains the photosynthetic activity under red light illumination was 80% to that of white light, since mainly PSI was excited. When illuminated with blue-green light which excites both photosystems, the photosynthetic oxygen production by transformant *-crtB* was found somewhat higher (96%) than in the other two strains (88%), which may indicate a small light-harvesting contribution of additional Cars in transformant *-crtB*.

Table III. Oxygen evolution of *Synechococcus* PCC7942 control (*-pFP1–3*) and transformant (*-crtB* and *-pys*) cells, illuminated with light of different quality, but of the same intensity (120 μEinstein m^{-2} s^{-1}). Rates are calculated in μmol O_2 x mg chlorophyll^{-1} x h^{-1} (A) or in μmol O_2 x ml pcv^{-1} x h^{-1} (B), and as a percentage of the rate of oxygen evolution by transformant cells illuminated with light of different quality, in relation to the rates obtained by illumination with white light (C).

Transformant	Light quality[a]			
	White	Red	Orange	Blue-green
A				
-pFP1–3 (control)	136 (100%)	110 (100%)	128 (100%)	122 (100%)
-crtB	153 (112%)	122 (111%)	136 (122%)	145 (119%)
-pys	162 (119%)	128 (116%)	159 (124%)	141 (116%)
B				
-pFP1–3	697 (100%)	565 (100%)	656 (100%)	614 (100%)
-crtB	1011 (145%)	824 (146%)	1039 (158%)	966 (157%)
-pys	873 (125%)	693 (123%)	855 (130%)	769 (125%)
C				
-pFP1–3	100	81	94	88
-crtB	100	81	103	96
-pys	100	79	98	88

[a] For spectral characteristics of different light beams see Materials and Methods.

Fluorescence induction

To examine a possible role of the additional Cars in light-harvesting, the activity of photosynthetic electron transport of the transformant cells was followed by measuring transient kinetics of variable fluorescence, which indicate the ratio between the rates of electron influx into the plastoquinone pool of PSII and the efflux of electrons to PSI (Krause and Weis, 1991). While the PSI/PSII ratio in higher plants is about unity, cyanobacteria are characterized by a higher content of PSI (Golbeck, 1992) that causes delay of fluorescence transients when mainly PSII is excited. If additional Cars were located in PSII antenna and fluorescence is excited by blue-green light, the steady-state level of variable fluorescence in Car-overproducing strains would be attained faster than in the control strain. In case of preferential location of Cars in PSI antenna the steady-state level of variable fluorescence of transformant cells would be delayed as compared to the control.

Fig. 3 shows very similar kinetics of variable fluorescence in the three transformants measured under excitation by blue-green light. The initial fast rise of fluorescence, ascribed to constant fluorescence (F_o), is followed by a slow enhancement of

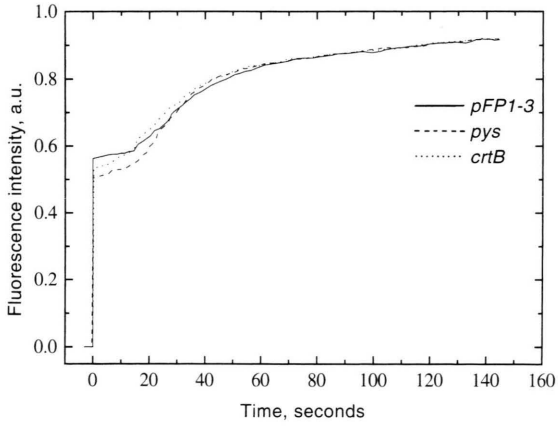

Fig. 3. Time courses of fluorescence induction of *-pFP1–3* control strain, *-pys* and *-crtB* transformant cells of *Synechococcus* PCC7942, measured at 298 K under excitation 400–500 nm light, with an intensity of 30 μEinstein m^{-2} s^{-1}. Kinetics curves are normalized for the maximum stationary signal; chlorophyll content 2.5 μg/ml (see text).

fluorescence to the steady-state fluorescence level (F_{max}). The fluorescence induction kinetics of all strains showed the same temperature dependence (19–29 °C); the addition of 0.1 mM DCMU slightly increased the initial fluorescence rise, in accordance with Mohanty and Govindjee (1973). Thus, no difference in the fluorescence induction kinetics of transformants *-crtB* or *-pys* was detected indicative of a higher activity of PSII of these strains.

Fluorescence spectra

A possible accumulation of overproduced Cars in the antenna of PSI or PSII core complexes was analyzed by measurements of 77-K fluorescence emission and excitation spectra of PB-free membranes. The main maximum at 716 nm (F716) emitted by PSI, and a band at 695 nm (F695) emitted by PSII appeared in the spectra with Chl (440 nm) or Car (500 nm) excitation (Fig. 4). A band at 685 nm is observed in the spectra of membranes from all strains under the excitation of Chl but not Car. When Chls were excited an additional shoulder at 670 nm was present in the emission spectra of the membranes from the *-pFP1–3* control strain (Fig. 4, A). One may speculate that this emission is due to uncoupled Chls since this band was not found in the spectra of transformants *-crtB* and *-pys* by excitation of Car which transfer energy mainly to coupled Chls. This band is visible in the emission spectrum of the control strain (*-pFP1–3*) even under Car excitation (Fig. 4, B).

The overlap of emission bands at 670, 685 and 695 nm in the *-pFP1–3* strain caused an increase of the intensity of F685 and F695. Taking this into account, there was no pronounced difference in the emission spectra of the membranes from different transformants, and the same value of the ratio F716/F695 could be observed for all strains. Under excitation of Car the ratio F716/F695 was found higher than that under excitation of Chls, since the main part of Cars is preferentially bound to PSI. This is not surprising since the PSI/PSII ratio is about 3–4 in cyanobacteria (Golbeck, 1992) and more Cars have to be located in PSI. Thus, the ratio between PSI and PSII was not changed in transformants containing a higher Car amount, although at the same time *-crtB* contains more Chls as compared with the two other strains (Table II). Apparently there are some changes in

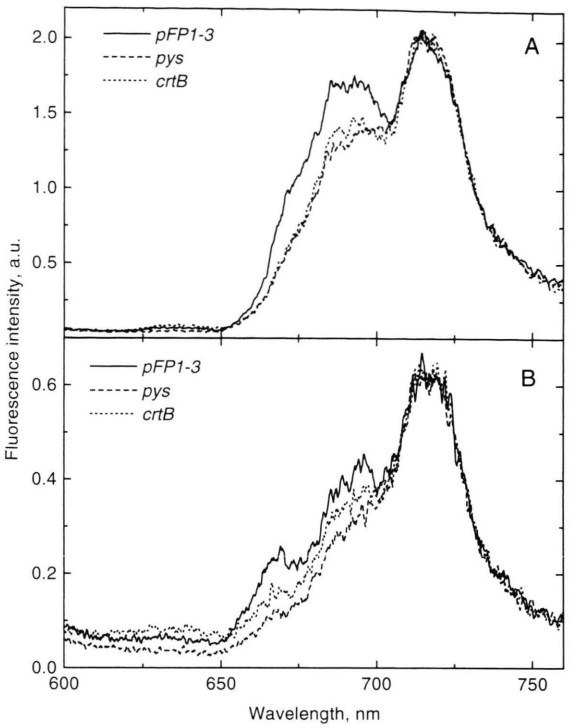

Fig. 4. 77-K fluorescence emission spectra of membranes isolated from the *-pFP1–3*, *-pys* and *-crtB* transformant cells of *Synechococcus* PCC7942; excitation wavelength 440 nm (A) or 500 nm (B). Spectra are normalized for 716 nm; chlorophyll content 0.5 μg/ml.

the state of antenna Chls of the two transformants compared to the control strain.

Clear bands at 470 and 505 nm due to Car absorption appeared in the PSI fluorescence excitation spectra of the transformants (Fig. 5). But the lower ratio between the intensities of these bands at 678 nm of the excitation spectra as compared with absorption spectra (Fig. 2) indicates that the energy migration efficiency from Cars to Chls in cyanobacterial membranes is less than 100%. The red maximum of excitation spectra of membranes from both transformants peaked at about 676 nm, while the red maximum of membranes from the control strain *-pFP1–3* peaked at about 673 nm which could be due to the absorption of some uncoupled Chls. Excluding this difference, the PSI fluorescence excitation spectra of all membranes in the spectral region measured are similar (Fig. 5). The PSII fluorescence excitation spectrum could not be measured due to the low intensity

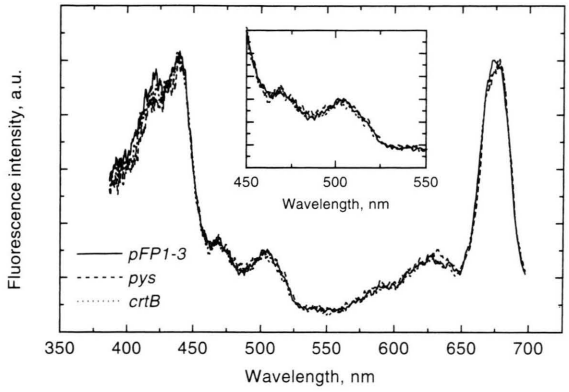

Fig. 5. 77-K fluorescence excitation spectra of PSI measured for the emission band at 725 nm of membranes isolated from *-pFP1–3* control strain, *-pys* and *-crtB* transformant cells of *Synechococcus* PCC7942. Spectra were measured for the same samples as used in Fig. 4 and normalized for the red maximum; chlorophyll content 0.5 μg/ml.
Inset: excitation spectra in the region of carotenoid absorption (450–550 nm).

emission of PSII antenna in cyanobacteria, and to the strong overlap of PSII and PSI emission bands.

Thus the PSI antenna of transformants is not enriched with Cars. Although *cells* of the transformants *-crtB* and *-pys* differ in their Car/Chl ratio (Table II), this ratio was found identical for the PSI complex of the (isolated) *membranes* (Fig. 5). It is suggested that overproduced Cars in the transformant cells are located in *non*photosynthetic membranes. Accordingly, light absorption by excess overproduced carotenoids results in a lower rate of oxygen evolution by *-pys* cells at saturating light intensity (Windhövel *et al.*, 1995).

Conclusion

Higher photosynthetic activity of transformants *-pys* and *-crtB*, which contain more Cars than the initial strain, was observed not only by the excitation of Cars but also by excitation of other pigments. The reason for the higher activity of these strains is not yet clear, since electron transport activity as reflected by fluorescence induction kinetics, was similar in all strains. This study shows that overproduced Cars are not included in the core antenna of PSI complexes which in cyanobacteria make up about 80% of the Chl-binding antenna (Golbeck, 1992). If the structure of PSI and PSII pigment-protein complexes is as highly conservative as the structure of LHCII (Hobe *et al.*, 1994), the overproduced Cars do likely bind neither to PSI, nor to PSII complexes. In comparison, the massive amounts of β-carotene accumulated by *Dunaliella* are located within small globules in the interthylakoid spaces of the chloroplasts (Ben-Amotz *et al.*, 1982). The protecting role of Cars for photosynthetic membranes and cells may be shown by future "bleaching" experiments examining the photosynthetic parameters of cyanobacterial cells and their growth after illumination with UV radiation or light of higher intensity than used in this study.

Acknowledgments

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