RESEARCH ARTICLE

Subcellular Localization of Carotenoid Biosynthesis in *Synechocystis* sp. PCC 6803

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Abstract

The biosynthesis pathway of carotenoids in cyanobacteria is partly described. However, the subcellular localization of individual steps is so far unknown. Carotenoid analysis of different membrane subfractions in *Synechocystis* sp. PCC6803 shows that "light" plasma membranes have a high carotenoid/protein ratio, when compared to "heavier" plasma membranes or thylakoids. The localization of CrtQ and CrtO, two well-defined carotenoid synthesis pathway enzymes in *Synechocystis*, was studied by epitope tagging and western blots. Both enzymes are locally more abundant in plasma membranes than in thylakoids, implying that the plasma membrane has higher synthesis rates of β-carotene precursor molecules and echinenone.

Introduction

Carotenoids are widely found in all oxygenic photoautotrophic organisms, generally embedded in their cellular membranes. Due to their chemical and physical properties, carotenoids influence diverse membrane properties, including fluidity and polarity [1]. They have important roles in protecting organisms against photo-oxidation and photo-inhibition [2–4]. This role is correlated with their ability to quench excited chlorophyll a (Chl a) triplet states which can generate toxic singlet oxygen [5]. Carotenoids are also believed to be essential in the synthesis, accumulation and maintenance of the integrity of the photosynthetic apparatus [6].

Cyanobacteria are a large and diverse group of oxygenic, photosynthetic bacteria. Different species and strains of cyanobacteria have different carotenoid compositions [7–9]; growth conditions, such as growth stage, light intensity [10,11], nitrogen source [12] and even the strain type within a given species can affect the types and amount of carotenoids as well. The major carotenoids in cyanobacteria are β-carotene, its hydroxyl derivatives, zeaxanthin and nostoxanthin, its keto derivatives, echinenone and canthaxanthin and the carotenoid glycosides, myxol-2'-glycosides and oscillol-2,2'-diglycosides. *Synechocystis* sp. PCC 6803 is a widely used model organism in many research areas, such as membrane organization, stress responses, metabolic pathways and recently also in synthetic biology studies. In *Synechocystis*, the major

carotenoid components are β-carotene (26%), myxoxanthophyll (36%), zeaxanthin (14%) and echinenone (18%), with other minor carotenoids comprising the remaining 6% [7].

The first committed step in carotenoid biosynthesis is the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to form phytoene (see Fig 1) [13]. In *Synechocystis* this reaction is catalysed by phytoene synthase, encoded by the *crtB* (*slr1255*) gene [6]. Phytoene is then converted to various carotenes that are substrates for the synthesis of a variety of xantho-phylls. Several enzymes have been identified and shown to be involved in the conversion of phytoene to β-carotene via lycopene. CrtP (Slr1254) is a phytoene desaturase synthesizing ζ-carotene, the deletion of which resulted in absence of β-carotene and its derivatives in *Synecho-cystis* [14]. ζ-carotene is further desaturated by CrtQ (Slr0940), a ζ-carotene desaturase that synthesizes lycopene [14–16]. Lycopene is the starting compound of various group modifica-tions that produce a large variety of carotenoids with different physical properties. So far, four types of lycopene cyclase have been identified in different groups of bacteria, CrtL [17], CrtY [18,19], heterodimeric CrtY cyclases [20], and CruA/CruP [21]. Though cyclic carotenoids have been detected in *Synechocystis*, the enzyme responsible for cyclization of lycopene to β–carotene has not yet been positively identified. Sll0254, for instance, was previously reported to function as a lycopene cyclase/oxygenase but no evidence was found for its catalysing β–caro-tene formation directly [22,23]. β-carotene is further modified by enzymes such as CrtO [24] and CrtR [25], to produce echinenone and zeaxanthin respectively. The *crtO* (*slr0088*) gene, encoding the β-carotene ketolase, was previously inactivated in *Synechocystis*, with the resulting ΔcrtO mutant being unable to synthesize echinenone [24].

β-carotene was found in the existing three-dimensional structures of photosynthetic com-plexes, with 12 molecules present in photosystem (PS)II [26], 22 in PSI [27] and one in the
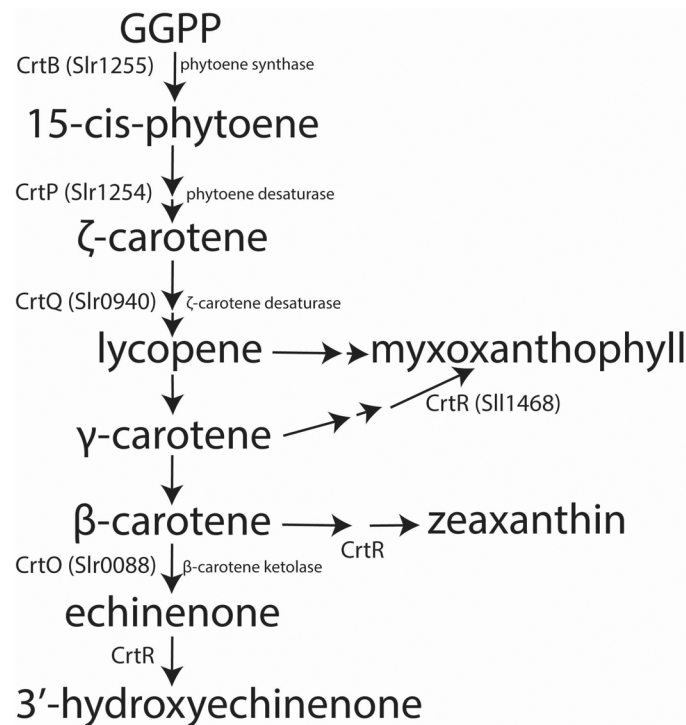

Fig 1. A simplified pathway for carotenoid biosynthesis in *Synechocystis*. Only enzymes with verified function are included.

doi:10.1371/journal.pone.0130904.g001

cytochrome (Cyt) b_6f complex [28]. It is the major carotenoid species in *Synechocystis*, with an important role in photoprotection and shown to be required for assembly of PSII but not for that of PSI [29]. Other photoprotective mechanisms exist in cyanobacteria, one involving the down-regulation of photosynthesis by increasing thermal dissipation of the energy absorbed by the PSII antenna. The orange carotenoid protein (OCP), binding 3'-hydroxyechinenone as well as echinenone, is the key player in this photoprotective reaction in cyanobacteria [30,31].

There are two distinct inner membrane systems in *Synechocystis*, the plasma membrane (PM) and the thylakoid membrane (TM). PM can be further separated into lipid-rich "light" PM (PM1) and the major PM (PM2) fraction, which has the same density as the thylakoid membrane. The light PM fraction can be obtained by sucrose density gradient, while the major PM fraction can only be derived from TM by several steps of aqueous 2-phase polymer separations [32]. Even though the proteome of the different membrane fractions has been studied intensively, the study of carotenoid distribution is rare, especially at the level of subcellular membrane fractions. In this study, we focused on characterizing the carotenoid composition of different membranes in *Synechocystis* and locating the two important pathway steps catalysed by CrtQ and CrtO.

Materials and Methods

Strains and growth conditions

Synechocystis sp. PCC6803 wild type (WT) and mutants were grown in BG11 medium, in 1L glass bottles at 30°C, with sterile air bubbling and at a light intensity of 50 μE m^{-2} sec^{-1}. Media were supplemented with 30 μg chloramphenicol ml^{-1} as required. Cell cultures with an OD_{730} between 1 and 1.5 were harvested by centrifugation at 8000 g and 4°C for 15 minutes, washed once in cold Lysis Buffer (20 mM potassium phosphate buffer, pH 7.8) and cell pellets were stored at -80°C until further use. For growth curve measurement, three independent WT and mutant cultures were grown to OD_{730} of 1.0, centrifuged at 3000 g for 15 min at room temperature and resuspended in fresh BG11 medium, without antibiotics, to a starting OD_{730} of 0.1. Cultures were incubated in a shaking incubator at 30°C, with constant overhead illumination at a light intensity of 50 μE m^{-2} sec^{-1} and shaking at 200 RPM.

Construction of CrtQ-FLAG and CrtO-FLAG *Synechocystis* strains

Genomic DNA from *Synechocystis* was isolated [33] and the *crtQ* (*slr0940*) gene, together with up- and down-stream sequences of roughly 1 kbp was amplified from it by PCR (Phusion DNA polymerase, New England Biolabs; see S1 Table for primer sequences). This sequence was cloned into the pCR-Blunt II vector (Invitrogen) following manufacturers' instructions, resulting in plasmid pCR-CrtQ. The 3xFLAG (hereafter, FLAG) epitope sequence was introduced in-frame at the 3' terminal of *crtQ* gene, before its stop codon, by restriction-free cloning [34,35] using a synthetic dsDNA megaprimer (see S1 Table), resulting in plasmid pCR-CrtQ-FLAG. A chloramphenicol (Cm) resistance cassette, derived from plasmid pSK9 (a kind gift from Annegret Wilde), was amplified with hybrid primers (sequences in S1 Table) and likewise inserted to the vector pCR-CrtQ-FLAG by RF cloning. This constructed plasmid pCR-CrtQ-FLAG-Cm^R was transformed into the wild type *Synechocystis* cells as described [36]. The *crtO* gene (*slr0088*) was cloned and epitope-tagged using a similar strategy, using the primers described in S1 Table. Positive clones were confirmed by PCR using total genomic DNA as template (S1 Fig).

Membrane fractionation

Synechocystis cell pellets from three independent 1 L cultures were washed once with chilled Lysis Buffer and resuspended in 1 mL of Lysis Buffer supplemented with protease inhibitors (Complete, EDTA-free, Roche). The cell suspension was lysed and membrane fraction preparation by sucrose density gradients/aqueous two-phase partitioning was performed according to previously established protocols [32,37]. Protein content was quantified by the Peterson method [38].

Protein electrophoresis and Western blotting

5 µg membrane proteins from each strain were separated on 12% TGX precast SDS-PAGE gels (BioRad) or homemade 12% SDS-PAGE gels supplemented with 7M urea (for photosystem components). Western blots were performed as previously described [32], with antibodies for typical thylakoid membrane (PsaA and CP43, Agrisera) and plasma membrane proteins (KtrE, obtained from Nobuyuki Uozumi). Anti-FLAG M2 antibody was purchased from Sigma for detecting tagged proteins. To characterize the membrane association of CrtO and CrtQ, cells from three independent cultures were lysed as described above and total membranes and soluble fraction separated by ultracentrifugation at 200000 g, for 1 hr, at 4°C, as previously described [32]. 200 µg of total membranes were centrifuged at 200000 g, for 20 min at 4°C and resuspended in 50 µL of Extraction Buffer 8 (EB8; 20 mM Tricine, pH 8.0), Extraction buffer 12 (EB12; 20 mM CAPS, pH 12.0) or EB8 supplemented with 6M urea (EB8+U). Samples were incubated as previously described [39]. Briefly, samples were subjected to two freeze/thaw cycles (30 min at -80°C and 20 min at 20°C) after which they were centrifuged at 200000 g, for 20 min at 4°C. Pellets were resuspended in 50 µL of EB8 buffer and 5 µL of each sample (pellet or supernatant) were analysed by SDS-PAGE and western blotting (polyclonal PsbO antibodies were a kind gift from Peter Nixon). Each blot was repeated at least twice. To investigate whether CrtO and CrtQ were part of stable membrane complexes, pull-down experiments with anti-FLAG M2 resin (Sigma-Aldrich) and Clear Native (CN) gels were performed using total membrane samples, followed by western blots, according to previously established protocols [40].

Carotenoid analysis by HPLC

Membrane fractions of *Synechocystis* cells were subjected to pigment extraction with two different methods, acetone:methanol (7/2, v/v) [41] and chloroform:methanol:water (1/1/0.8, v/v) [42]. In both cases the pigment extracts were dried under a stream of nitrogen and re-dissolved in ethanol. Both carotenoid preparations were separated by reversed-phase high-performance liquid chromatography (HPLC) at 15°C, with a flow rate of 1 mLmin^{-1} on a Purospher STAR RP-18 endcapped column, 150 mm x4.6 mm (Merck).

The pigment separation started with a 4 min isocratic elution with solvent A system of acetonitrile:methanol:Tris (0.1M, pH 8.0) (89/9.5/1.5, v/v), followed by a 2.5 min linear gradient of solvent B, methanol:hexane (4/1, v/v). Solvent B continued isocratically for 11.5 min before a linear gradient of solvent A for 1 min, and a final isocratic elution with solvent A for 9 min. The total run time was 28 min. Carotenoids were detected at 440 nm. Pigments were identified by comparison of the known carotenoid composition of *Synechocystis* [43], the HPLC retention times, absorption spectra and commercially available zeaxanthin, β-carotene (Extrasynthèse, www.extrasynthese.com), myxoxanthophyll and echinenone (DHI www.labproducts.dhigroup.com) standards. The concentration of zeaxanthin, β-carotene, myxoxanthphyll and echinenone was quantified based on the peak area of known standards (Extrasynthèse and DHI). The minor component γ-carotene was quantified as β-carotene and hydroxyechinenone and cryptoxanthin were quantitated as zeaxanthin. Three independent membrane preparations

were used for the carotenoid analysis, and each sample was analyzed typically 3 times. The handling of pigment samples was performed in dim light.

Pigment composition analysis by absorption scanning

Pigment composition was compared between WT and FLAG-tagged strains by performing absorption scans on exponentially grown cells in BG11 medium as previously described [44]. Cells were diluted with BG11 to an OD_{730} of ≈ 0.22 and absorption was measured from 380 to 730 nm using a Cary 300 Bio UV-Visible Spectrophotometer (Varian). The spectra obtained (S3 Fig) were normalized to the absorption at 730 nm.

Results

Light plasma membrane has the highest carotenoid/protein ratio, while the heavy plasma membrane has the lowest

Carotenoid extraction from PM1, PM2 and TM of wild type cells was carried out with two different extraction methods. Similar results were obtained with both methods and included in the mean of each independent sample. Substantial differences were observed between different membranes, with the total content of carotenoids on a protein basis being highest in PM1, while in TM and PM2 there were only 16% and 11% respectively of the PM1 carotenoid content (Fig 2A). The carotenoid composition analyses revealed that each membrane has the same kind of carotenoids though in different proportions (Fig 2B). For instance, in PM1, 54% of the total carotenoid content is comprised of β-carotene, with myxoxanthophyll, zeaxanthin, echinenone and other minor carotenoids. This character is similar to that of TM (the most
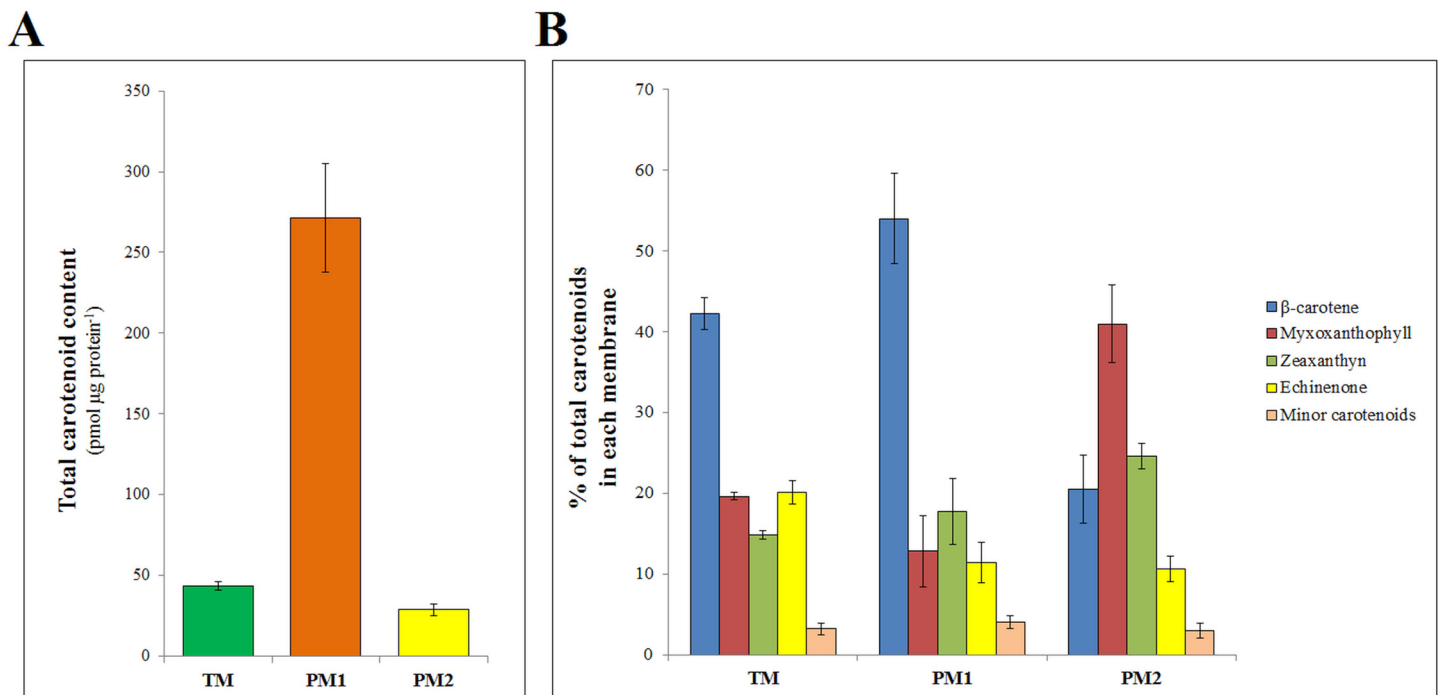

Fig 2. Carotenoid distribution in *Synechocystis* membranes. Total carotenoid amount in purified PM1, PM2 and TM (A). Relative amount of different carotenoids in each membrane (B). Data represent means ± standard deviations for carotenoids in membranes from three independent cultures. For results of statistical analysis, please refer to S2 Table.

abundant membrane in *Synechocystis*), which also contains mostly β-carotene, albeit in a lower concentration (42%). PM2, on the other hand, has a different composition in comparison to other membranes, with 41% of myxoxanthophyll, 25% of zeaxanthin and 20% of β-carotene.

CrtQ and CrtO are more concentrated in plasma membrane than thylakoid membrane

To understand the cause for this unequal carotenoid distribution in *Synechocystis* membranes, the distribution of enzymes involved in carotenoid biosynthesis was further investigated. Two of these enzymes, CrtQ and CrtO, were chosen due to their well proven functions. As suitable antibodies are unavailable, 3xFLAG epitopes were added to the C-terminal end of each protein, which were under the control of their original promoters and in the original loci. The strains thus obtained did not show measurable pigment and growth differences (see S2 and S3 Figs). PM1, PM2 and TM were isolated from CrtQ-FLAG and CrtO-FLAG cells and the purity of the membrane fractions were confirmed with antibodies against PsaA, CP43 and KtrE (Fig 3A). Anti-FLAG blots showed single specific band in both strains, yet with slight differences regarding their distribution. In the case of CrtQ, the highest concentration of the FLAG signal could be detected in PM1 and PM2, with similar amounts in these two membranes (Fig 3). At the same time, only about 30% of the amount in PM1 could also be found in TM. On the other hand, our data showed that CrtO is strongly concentrated in PM, with similar distribution between PM1 and PM2. To get a similar intensity for both proteins, roughly 50 times longer exposure was needed for CrtO (3 min vs 4 sec), indicating that CrtO was less abundant than CrtQ.

No large complexes containing CrtQ or CrtO proteins observed in *Synechocystis* membranes

Both CrtQ and CrtO are predicted to be soluble proteins, with some hydrophobic regions in the N-terminal, by online softwares TOPCONS (www.topcons.net) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). These proteins are involved in the synthesis of carotenoid molecules and it is expected, from the hydrophobic nature of their products, that they should strongly associate to the membrane. Total membranes were washed with both high pH and high concentrations of urea, so as to remove membrane associated proteins, and anti-FLAG western blots of the resulting fractions showed that, though also present in the soluble fraction, neither membrane-associated CrtO nor CrtQ could be completely removed by either high pH or urea treatment, in opposition to the luminal PSII subunit PsbO (Fig 4A).

To clarify whether these proteins form part of large protein complexes, associate to the photosystems or are involved in other integral membrane complexes in *Synechocystis*, we attempted to isolate CrtO- and CrtQ-containing complexes by pull-down with anti-FLAG resin and analysis of the resulting eluates in CN-PAGE/western blots. In both cases we were unable to detect high molecular weight complexes containing either CrtO or CrtQ (Fig 4B). CrtQ could be seen mostly in the form of free protein (Fig 4B). CrtO, though the signal is much weaker, could also be seen to migrate in the low molecular range of the gel, while no signal was observed in the WT pulldown sample. A second slower migrating band could be observed in both cases, though its nature could not be determined by western blot at this moment.

Discussions

Where does carotenoid biosynthesis occur in Synechocystis?

Carotenoid biosynthesis in cyanobacteria is not as well characterized as in plants. In *Synechocystis*, for instance, only a handful of enzymes (CrtB, CrtP, CrtQ, CrtO and CrtR) have had
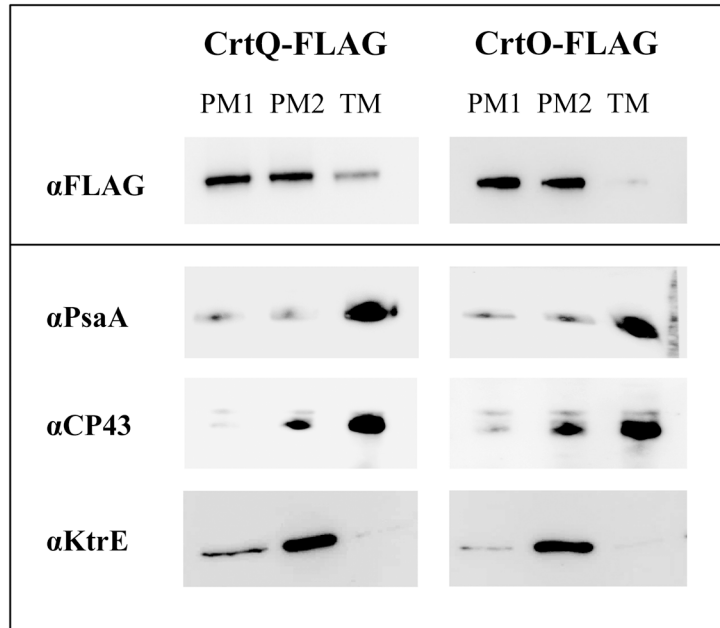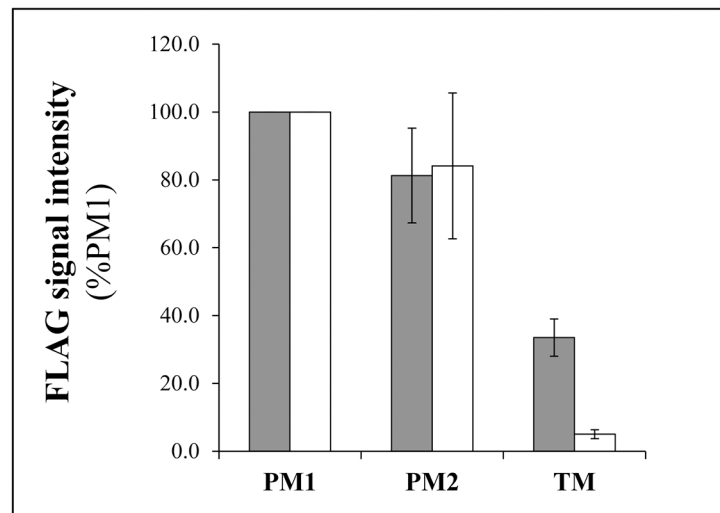
Fig 3. CrtQ and CrtO localization. Western blots analyses of purified PM1, PM2 and TM from CrtQ-FLAG and CrtO-FLAG strains. Exposure times were 4 and 180 seconds respectively (A). Quantifications of CrtQ (grey) and CrtO (white) in each membrane, based on four independent western blots (B). Data represent means ± standard deviations for four independent quantifications. For results of statistical analysis, please refer to S2 Table.

doi:10.1371/journal.pone.0130904.g003

their function experimentally proven (Fig 1) [6,14–16,24]. Furthermore, their respective intracellular distribution has not yet been established. Our results show that CrtQ, one of the enzymes involved in the early steps of β-carotene formation, has a higher local concentration in PM than in TM (Fig 3). The carotenoid quantification data showed highest pigment/protein ratios in PM1 (Fig 2A). Since TM is the most abundant membrane and PM1 only constitutes a
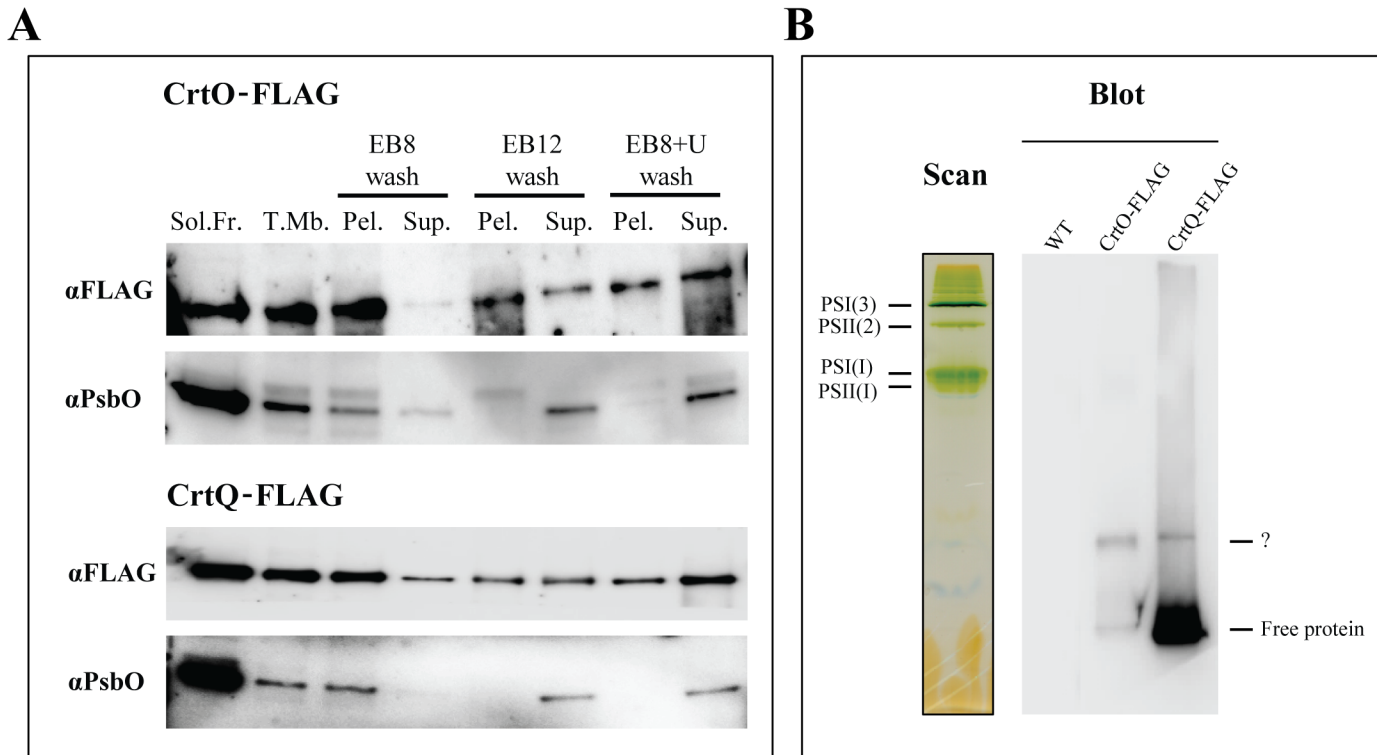
Fig 4. Membrane binding analyses of CrtQ-FLAG and CrtO-FLAG. Western blots of soluble fractions vs. total membranes (5 µg each) isolated from both strains and the effect of different washes on CrtO and CrtQ membrane association. Total membranes were washed with EB8, EB12 or EB8+U and soluble ("Sol.") and pellet ("Pel.") fractions were generated by ultracentrifugation. Each lane was loaded with equal volumes of (resuspended) pellet or supernatant fractions. Three independent cultures were tested in this manner. (A). Western blots analysis of anti-FLAG resin eluates from 2% DDM-solubilized total membranes of WT, CrtO-FLAG and CrtQ-FLAG cells, separated on 4–18% Clear Native–PAGE (B). Colour scan of CN-PAGE gel for WT total membrane showing the relative migration of several known complexes.

doi:10.1371/journal.pone.0130904.g004

very small part, the majority of carotenoids present in the cell exist in the TM. At the same time, over 40% of the total carotenoid accumulated in TM is β-carotene ([Fig 2B]). The large proportion of this pigment in the light plasma membrane fraction (PM1) and the high local concentration of the enzyme (CrtQ) responsible for synthesis of its precursor could reflect a local heterogeneity in the plasma membrane. Lycopene does not accumulate to any measurable extent in *Synechocystis*; therefore, the conversion between lycopene and β-carotene must be fast and complete. As such, though we are only able to locate the enzyme responsible for lycopene synthesis, it seems logical to consider that PM1 could be the major synthesis site of lycopene and, due to this fast conversion between the two, of β-carotene as well.

In addition, CrtO, which is the enzyme catalysing the formation of echinenone and 3-hydroxyechinenone in the late stage of carotenoid synthesis, is also strongly concentrated in PM but not in TM ([Fig 3]). Both echinenone and 3'-hydroxyechinenone loading are important for functional OCP [30,31]. It was previously shown that OCP strongly interacts with the thylakoids, acting as a quencher to dissipate the excess energy arriving at the reaction centres [45]. Our results indicate that a higher local synthesis of echinenone and 3'-hydroxyechinenone should take place in PM. Whether the pigment loading to OCP happens in PM or echinenone/3'-hydroxiechinenone diffuse to TM prior to OCP loading still remains to be ascertained.

Owing to the scarcity of data on the identity of carotenoid synthesis enzymes in *Synechocystis*, we chose to study the location of these two particular enzymes. CrtQ is responsible for the synthesis of lycopene, the step just prior to conversion to β-carotene, thus serving as a means to locate early carotenoid biosynthesis steps. CrtO, on the other hand, catalyses the synthesis of a pigment after the branching point of the carotenoid pathway ([Fig 1](#)), essential for non-photochemical quenching. However, both of these enzymes seem to be much more locally concentrated in the plasma membrane and, together with the strikingly high carotenoid/protein ratio in PM1, it is tempting to point to PM, and especially to PM1, as being the major site of carotenoid synthesis in *Synechocystis*. CrtO, in particular, is especially concentrated in the PM fractions ([Fig 3](#)). Further research will be required to unambiguously identify other enzymes within the pathway (for instance, the glycosyltransferase required for myxoxanthophyll synthesis), at which point we shall be able to confirm out hypothesis. However, an interesting parallel can be established here with another cyanobacterium, *Gloeobacter violaceus*. This unusual cyanobacterium does not have separate thylakoid membranes but, instead, discrete domains within its plasma membrane where photosynthetic complexes are located [46]. This "green fraction" can be isolated from the "orange fraction" by biochemical methods and the composition of both fractions was previously studied in detail. It was concluded that these different fractions or domains are formed by lateral heterogeneity and phase transitions induced by their very different protein and pigment composition, rather than differences in lipid species, as would be the case of lipid rafts. The "orange fraction" contains more carotenoids than the "green fraction", which had 20 times more chlorophyll but only 4% of the carotenoid content [46]. Furthermore, an enzyme of the carotenoid biosynthesis pathway—phytoene desaturase or CrtI—was found exclusively in the "orange fraction" [46] and it was suggested that the carotenoid synthesis-related proteins should therefore accumulate in the "orange fraction". It is tempting to see the PM1 fraction of *Synechocystis* as being very similar to this "orange fraction" of *G. violaceus*—laterally heterogeneous fractions of the membrane where enzymes responsible for carotenoid synthesis and their respective products accumulate and where, perhaps, the synthesis rate of these compounds is the highest.

β-carotene biosynthesis does not directly interact with the photosynthetic complexes

Carotenoids are an intrinsic part of photosystem structures, as previously mentioned, and it was proposed that their synthesis regulates PSII assembly [6]. As such, it was important to understand whether CrtQ would form membrane complexes, either with PSII assembly precursors or with other (putative) proteins, acting as mediators in PSII β-carotene loading. Our results show that CrtQ exists in a considerable amount in TM (30% of the amount in PM, [Fig 3](#)), but it does not seem to form high molecular weight complexes with PSII or other assembly intermediates, being present mostly as a free protein ([Fig 4B](#)). A weak band could also be seen above the free CrtQ band, though its composition is unknown for the moment. This is in opposition to what is described in higher plants, where the carotenoid synthesis enzymes apparently form a large membrane-associated complex [47] Thus, it is likely that lycopene formed by CrtQ flows freely into the membrane, from where it is then inserted into the photosystem complexes when required. We were also unable to identify any large complexes containing CrtO. Though the association of these two proteins to the membrane is quite strong (see [Fig 4A](#)), so far we were unable to positively identify interacting membrane protein partners that would stabilize them in a high molecular weight complex. As such, it may be that their association to the membrane is mostly dependent on hydrophobic interactions, either with the lipid or pigment components.

Supporting Information

S1 Fig. 0.8% agarose gel of segregation screening PCR for FLAG-tagged CrtO and CrtQ *Synechocystis* **strains.** Segregation screening PCR was performed using primers indicated in S1 Table.
(TIF)

S2 Fig. Growth curves for WT and FLAG-tagged CrtO and CrtQ *Synechocystis* **strains.** OD_{730} data points are the average of three independent cultures, with error bars representing the standard deviation.
(TIF)

S3 Fig. Representative whole cell absorption scans of WT and FLAG-tagged CrtO and CrtQ *Synechocystis* **strains.**
(TIF)

S4 Fig. HPLC chromatogram of a typical PM1 pigment extract. Peaks were detected by absorbance at 478 nm. Labelled peaks correspond to myxoxanthophyll (1), zeaxanthin (2), hydroxyechinenone (3), echinenone (4), cryptoxanthin (5), β-carotene (6) and γ-carotene (7). Chlorophyll retention time under these conditions was 11.2 minutes (chlorophyll peak is not visible at 478 nm).
(TIF)

S1 Table. Primers used in construction of FLAG-tagged CrtO and CrtQ *Synechocystis* **strains.**
(DOCX)

S2 Table. P-values for Student's t-test analysis of quantifications shown in Figs 2 and 3. $P<0.05$ was considered significant.
(XLSX)

Acknowledgments

We thank Gunilla Malmberg for skilful technical assistance.

Author Contributions

Conceived and designed the experiments: LZ TTS ES BN. Performed the experiments: LZ TTS ES. Analyzed the data: LZ TTS ES BN. Contributed reagents/materials/analysis tools: LZ TTS ES BN. Wrote the paper: LZ TTS ES BN.

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