Identification of a Carotenoid-Binding Protein in the Cytoplasmic Membrane from the Heterotrophic Cyanobacterium *Synechocystis* sp. Strain PCC6714

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We isolated a carotenoid-binding protein from the cytoplasmic membrane of the cyanobacterium *Synechocystis* sp. strain PCC6714. The polypeptide demonstrated a characteristic mobility shift when electrophoresed in lithium dodecyl sulfate-polyacrylamide gels. The protein migrated with an apparent molecular mass of 35 kilodaltons when solubilized at 0°C, but after solubilization at 70°C, the protein migrated as a 45-kilodalton species. The carotenoid-binding protein accumulated only in autotrophically grown cells; cytoplasmic membranes prepared from photoheterotrophically grown cells lacked this component.

Cyanobacteria are photosynthetic procaryotes which contain external and internal membrane systems that are structurally and functionally distinct. Like all gram-negative organisms, the cyanobacterial cell is delimited by a double-membrane envelope (5, 16), although the peptidoglycan is a dense, highly cross-linked structure more similar to the gram-positive cell wall (11). Within the cytoplasm are concentric layers of chlorophyll-binding thylakoid membranes which serve as the site for photosynthetic electron transport and ATP synthesis (7). Recent work on *Anacystis nidulans* and *Synechocystis* sp. strain PCC6714 described methods for the separation of the cytoplasmic membrane from both the cell wall and the thylakoids (15, 16). Purified cyanobacterial cytoplasmic membranes are intensely yellow-orange because of the presence of carotenes and xanthophylls (12, 15, 16). In this study, we identify a carotenoid-binding protein which is a major component of the *Synechocystis* sp. strain PCC6714 cytoplasmic membrane. This species strain is a unicellular cyanobacterium capable of both autotrophic and photoheterotrophic growth; under photoheterotrophic conditions, the cells grow in the presence of glucose and the photosystem II inhibitor, atrazine. Photoheterotrophic cells, which are incapable of producing oxygen, utilize glucose as a carbon source and light as an energy source, driving cyclic photophosphorylation around photosystem I (4). In this study, we show that the carotenoid-binding protein accumulates only in autotrophically grown, oxygen-evolving *Synechocystis* sp. cultures.

Synechocystis sp. strain PCC6714 *str-5* (3), a spontaneous mutant resistant to streptomycin (25 µg/ml), was grown in BG-11 medium (1) supplemented with NaNO₃ to 3 g/liter. For photoheterotrophic growth conditions, glucose and atrazine were added to final concentrations of 1% and 5 µM, respectively. Cells were grown with constant illumination (0.5 mW/cm²) and aeration at 25°C in 15-liter carboys. Alternatively, for the comparison of autotrophically and heterotrophically grown membranes small-volume cultures were grown in 200-ml volumes with aeration under the same light conditions.

Isolation of the carotenoid-binding protein. Cytoplasmic membranes were isolated essentially by the method of Omata and Murata (15), except that 50 mM morpho-

lineethanesulfonic acid (MES) (pH 6.5) was used to buffer all solutions. All buffers included the protease inhibitors benzamidine, phenylmethylsulfonyl fluoride, and ε-amino caproic acid, each at 1 mM final concentration. We believe that our cytoplasmic membrane preparation was free of contaminating cell wall material because this fraction lacked periodic acid-Schiff-staining material which would identify contaminating lipopolysaccharide (21). In addition, all procedures were carried out under conditions in which the gram-negative outer membrane remained insoluble (17, 18). Finally, our preparation lacked polypeptides of the molecular mass range which were shown to be major constituents of the *Synechocystis* sp. cell wall (12, 16). The carotenoid-binding protein was isolated by first pelleting membranes prepared from 5 liters of autotrophically grown culture (10 h at 115,000 × g in a Beckman 60 Ti rotor) and then suspending the pellet in 1.5 ml of 50 mM MES (pH 6.5) containing 0.2% Triton X-100 and 0.2% dodecyl-β-D-maltoside (Calbiochem-Behring). The membrane suspension was passed through an Affi-gel 501 organomercury column (Bio-Rad Laboratories; 1-by-15-cm bed) equilibrated in the same buffer. The orange material which eluted in the void volume was collected, pelleted, and suspended as described above. The Affi-gel 501 eluate was next applied to a DEAE-Sephacel column (1-by-25-cm bed equilibrated in MES-Triton X-100-dodecyl-β-D-maltoside) and washed with 4 volumes of the same buffer, containing 10 mM NaCl. Next, the column was washed with 2 volumes of buffer containing 100 mM NaCl before elution of the carotenoid-binding protein with 350 mM NaCl. The carotenoid-binding protein eluted from the column as a sharply defined orange band; portions of the orange fractions were retained for spectral and electrophoretic studies. Visible absorption spectra were obtained at 20°C with a Beckman DU-7 high-speed spectrophotometer. Photosystem II activity was measured by monitoring O₂ evolution, with FeCN and dichlorobenzoquinone as electron acceptors (8). Polyacrylamide gel electrophoresis in the presence of lithium dodecyl sulfate was performed by the method of Guikema and Sherman (9). Portions of membrane samples were solubilized at either 0 or 70°C before loading onto slab gels, because comparison of polypeptide patterns at the two solubilization temperatures often revealed banding pattern changes associated with ligand-binding species (3). Protein concentration was determined

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FIG. 1. Analysis of the carotenoid-binding protein, the cytoplasmic membranes, and the thylakoids by electrophoresis in 10 to 20% acrylamide gels. Lanes: 1 and 2, purified carotenoid-binding protein solubilized at 0 and 70°C; 3 and 4, *Synechocystis* sp. cytoplasmic membranes prepared from a 15-liter carboy solubilized at 0 and 70°C; 5 and 6, thylakoid membranes solubilized at 0 and 70°C. Electrophoresis was performed on lithium dodecyl sulfate-polyacrylamide gels (9), and the gel was stained with Coomassie brilliant blue.

by the method of Markwell et al. (14). Gels were silver stained by the procedure of Wray et al. (20).

Characterization of the carotenoid-binding protein. The orange material which eluted from the DEAE-Sephacel column contained a single polypeptide after Coomassie brilliant blue staining (Fig. 1, lanes 1 and 2). Two pieces of evidence suggest that the orange pigment specifically binds the protein. First, under nondenaturing conditions, the DEAE-prepared orange pigment and protein eluted together from Sephadex G-50. Second, the orange material could be pelleted through 10% sucrose when centrifuged at $115,000 \times g$ for 10 h. Free carotenoid pigments cannot be pelleted under these conditions. Both the orange column eluate and the pelleted fraction contained a single polypeptide. The protein demonstrated a characteristic shift in electrophoretic mobility when denatured at 70°C compared with denaturation at 0°C. When solubilized at 0°C, the polypeptide migrated with an apparent molecular mass of 35 kilodaltons (kDa), but the polypeptide shifted to a 45-kDa species after solubilization at 70°C (Fig. 1, lanes 1 and 2). During electrophoresis at both of these temperatures, orange pigment dissociated from the protein and ran as an orange band at the gel front. Comparison of electrophoretic profiles of cytoplasmic and thylakoid membranes prepared from autotrophically grown cells showed that the carotenoid-binding protein was a major polypeptide only in the cytoplasmic membrane fraction (Fig. 1, lanes 3 to 6). Furthermore, verification that the isolated carotenoid-binding protein and the major cytoplasmic membrane protein are the same component was determined by immunoblotting studies (19). A 46-kDa protein from a carotenoid-containing fraction of *A. nidulans* R2 was shown to be immunologically related to the *Synechocystis* sp. carotenoid-binding protein. A polyclonal antibody prepared against the *Anacystis* protein cross-reacted both with the isolated *Synechocystis* sp. polypeptide and with the 45-kDa polypeptide in heat-solubilized cytoplasmic membrane samples (H. C. Riethman, G. S. Bullerjahn, and L. A. Sherman, unpublished data).

The absorption spectrum of the native carotenoid-binding protein at 25°C revealed a characteristic carotenoid spectrum having three peaks at A_{450} , A_{476} , and A_{516} (Fig. 2A). After extraction of the pigment with 95% acetone, the spectrum yielded two peaks at A_{451} and A_{476} (Fig. 2B). The chemical structure of the carotenoid pigment associated with the protein has not been determined with certainty; however, D. W. Krogmann (personal communication) has obtained a spectrally identical preparation from *Synechocystis* sp. obtained after extraction with 0.5% cholate. Spectral

FIG. 2. Visible absorption spectra of the purified carotenoid-binding protein. (A) Absorption spectrum of the native protein after elution from DEAE-Sephacel with 350 mM NaCl. (B) Absorption spectra of the acetone-extracted pigments from the 350 mM eluate.

FIG. 3. Polypeptide composition of cytoplasmic membranes from 200-ml cultures of heterotrophic cells (lanes 1 and 2) and autotrophic cells (lanes 3 and 4). Samples were run on 10 to 20% acrylamide gels; the gel was silver stained (18). Note that the electrophoretic pattern of lanes 3 and 4 is not identical to that of lanes 1 and 2 in Fig. 1. This is most likely because of the differences resulting from the membrane preparation from small-volume cultures.

analysis indicated that the orange pigment is dehydroescholtzanthin, although mass spectrometry data are not yet available to confirm the structure of the carotenoid (D. W. Krogmann, personal communication).

Accumulation of the carotenoid-binding protein. Carotenoids have been implicated as agents which protect cells from photooxidative damage. For example, the white-3 mutant of maize, defective in carotenoid synthesis, cannot accumulate chlorophyll in the presence of both normal light levels and atmospheric oxygen concentration. However, the mutant does not bleach under conditions of low light or in a nitrogen atmosphere (2). This suggested that the mutant lacks carotenoid pigments which act to protect the photosynthetic apparatus in the presence of oxygen and chloro-

phyll, the photosensitizing agent. Such studies have also been applied to *Rhodospseudomonas sphaeroides* mutants lacking carotenoid, with similar results of oxygen sensitivity in light (6).

To investigate whether this polypeptide may play such a role, we compared the polypeptide composition of cytoplasmic membranes prepared from autotrophic and photoheterotrophic *Synechocystis* sp. cultures. *Synechocystis* sp. cells grown under photoheterotrophic conditions were incapable of oxygen evolution ($<10 \mu\text{M O}_2$ evolved per mg of chlorophyll per h) because of the presence of $5 \mu\text{M}$ atrazine in the medium, whereas autotrophic cultures evolved oxygen at the rate of $280 \mu\text{M O}_2$ evolved per mg of chlorophyll per h. Cytoplasmic membranes were isolated from these cultures and analyzed by visible absorption spectroscopy and electrophoresis. These comparisons revealed several differences between membranes prepared from the two culture conditions. First, the total amount of carotenoids in heterotrophically grown cells was considerably lower than that in autotrophically grown cells. With an absorption coefficient ($E_{1\text{cm}}^{1\%}$) of 2,500 (read at the middle main absorption maximum of the carotenoid spectrum), the carotenoid content of cytoplasmic membranes was estimated (13). Although the protein content of the membranes remained similar, autotrophic cytoplasmic membranes yielded a ratio of $240 \mu\text{g}$ of carotenoid per mg of protein, whereas heterotrophic membranes contained $62 \mu\text{g}$ of carotenoid per mg of protein. Second, the polypeptide composition of silver-stained cytoplasmic fractions isolated from small-volume cultures showed that the carotenoid-binding protein described here accumulated only in autotrophic cells (Fig. 3, lanes 3 and 4), not in heterotrophic cells (Fig. 3, lanes 1 and 2). Furthermore, the visible absorption spectra of these membrane preparations revealed that the autotrophic membranes had an absorption band at 516 nm which was absent in the heterotrophic membrane spectrum (Fig. 4). The 516-nm peak matched the long-wavelength band of the native isolated carotenoid-binding protein. The large spectral differences between the two membrane preparations may also indicate other changes in pigment composition.

In this study, we isolated a carotenoid-binding protein which is a major constituent of the *Synechocystis* sp. strain PCC6714 cytoplasmic membrane. We believe that this protein is distinct from the polypeptide described by Holt and Krogmann (10). Their preparation contained a 16-kDa

FIG. 4. Visible absorption spectra of cytoplasmic membrane preparations from photoheterotrophic (A) and autotrophic (B) cultures. The long-wavelength peaks at 672 nm are due to small amounts of chlorophyll contaminating the preparation. Carotenoid content was estimated by the procedure of Liaaen-Jensen and Jensen (13); measurements of the A_{457} and the A_{482} were used for heterotrophic and autotrophic cultures, respectively.

apoprotein characterized by denaturing electrophoresis and was present in aqueous extracts of *Spirulina maxima*, *Aphanizomenon flos-aquae*, and *Microcystis aeruginosa*. We do not know whether an analogous protein exists in *Synechocystis* sp. It is very likely that other carotenoid-binding proteins are present in the cytoplasmic membrane; perhaps these polypeptides are the species which show electrophoretic band shifts after solubilization at 0 and 70°C (Fig. 1 and 3). Furthermore, the fact that the spectra of Fig. 2A and 4B are not identical suggests the presence of other carotenoid-binding proteins in the cytoplasmic membrane. The protein described here accumulates in autotrophically grown cells and binds enough pigment to represent a major component of the visible absorption spectrum of the cytoplasmic membrane. The observation that this polypeptide is present in substantial quantities only in oxygen-evolving cultures suggests that it may play a role in photooxidative protection in the cell. Since there are very few identified proteins known to be associated with the cytoplasmic membrane, this polypeptide will be a useful molecular marker for studies of cytoplasmic membrane biogenesis; currently, we are preparing a homologous antibody to this protein so that we may detect its accumulation during autotrophic growth.

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