Isolation and Characterization of a Carotenoid-Associated Thylakoid Protein from the Cyanobacterium *Anacystis nidulans* R2

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**ABSTRACT**

A carotenoid-associated membrane protein was isolated from *Anacystis nidulans* R2 thylakoids. Sodium pyrophosphate and sodium bromide washed thylakoids were solubilized with the nonionic detergents dodecyl-β-D-maltoside and octyl-β-D-glucopyranoside, and these detergent extracts were fractionated on a sucrose density gradient. A yellow fraction from the sucrose gradient was further purified by anion-exchange and organomercuric-affinity column chromatography to yield a fraction virtually free of chlorophyll and highly enriched in both carotenoids and a 42 kilodalton polypeptide. Evidence presented in this paper suggests that the carotenoid-containing 42 kilodalton protein is thylakoid-associated rather than cytoplasmic membrane associated. The purified 42 kilodalton polypeptide was used to raise polyclonal antibodies in rabbits. Immunochemical detection of the 42 kilodalton polypeptide on Western blots demonstrated an increased accumulation of this polypeptide in cells grown under high-light conditions relative to cells grown under low light.

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Cyanobacteria have three distinct membrane systems; outer and inner (cytoplasmic) membranes comprise the cell envelopes, as in other gram-negative bacteria (8), whereas the internal thylakoid membrane system contains the components responsible for an oxygen-evolving electron transfer system unique among prokaryotes (10). Using improved isolation techniques, envelope (outer and cytoplasmic) membranes have been separated from thylakoids in both *Anacystis nidulans* (11, 22, 24, 27) and *Synechocystis* PCC6714 (10, 11, 17, 18), and have been shown to contain carotenoids but little or no Chl. The major carotenoids of the isolated outer and cytoplasmic membranes were xanthophylls, whereas the major thylakoid-associated carotenoid was β-carotene (11, 17, 18, 22, 24, 25, 27).

Analysis of *A. nidulans* membrane components involved in photosynthesis has yielded important information regarding integral membrane proteins that are stably liganded to Chl (3, 26). These Chl-binding proteins include the PSI Chl-protein and oligomers CP I, CP II, CP III, and CP IV (3), and the PSII-associated Chl-proteins CP VI-2 (spinach chloroplast CP 47 analog), CP VI-3 (spinach chloroplast CP 43 analog) and CP VI-4 (a Chl-protein synthesized in response to iron stress). An additional PSII-associated Chl-protein, CP VI-1, contains both the 71 kD phycobilisome ‘anchor’ polypeptide and somewhat variable amounts of an unidentified 42 kD polypeptide (26; HC Riethman, LA Sherman, unpublished data). The presence of carotenoids in the photosynthetic membranes of *A. nidulans* is likely to be physiologically important both for the protection of these Chl-proteins from photodestructive damage and for the harvesting of light energy in spectral regions where Chl a and phycobilisomes do not absorb strongly.

Although carotenoids have been found as pigments on many isolated Chl-binding proteins (32), there have been few reports of carotenoids specifically associated with isolated proteins in the absence of Chl. Holt and Kroppmann (14) reported on a carotenoid-binding protein isolated from aqueous extracts of several cyanobacteria, whereas Bullerjahn and Sherman (6) have isolated an integral membrane, carotenoid-associated protein from cytoplasmic membranes of *Synechocystis* PCC6714. The carotenoid-associated protein from *Synechocystis* PCC6714 was shown to have an immunologically related analog in *A. nidulans* R2 (5, 6). In this study we describe the isolation and characterization of a second carotenoid-associated protein from *A. nidulans* R2, one that is immunochemically distinct from that described previously (5, 6). This protein appears to be embedded in the thylakoid membrane, and accumulates specifically in cells grown under high light conditions.

**MATERIALS AND METHODS**

**Cell Growth.** *Anacystis nidulans* R2 was grown in BG-11 medium with constant illumination (0.5 mW/cm2) and aeration at 25°C in 15 L carboys as described previously (12). Growth under high white light and low white light were at intensities of 1.9 mW/cm2 and 0.02 mW/cm2, respectively. For preparation of thylakoids from iron-deficient cells, cells were grown in low-iron medium as described earlier (13, 29).

**Isolation of Thylakoid Membranes and the Carotenoid-Associated Membrane Protein Fraction.** Thirty L of *A. nidulans* cells were harvested at logarithmic phase (the approximate wet weight of harvested cells was 17 g), then washed once and suspended in 25 ml of buffer A (50 mM Mes [pH 6.5], 1 mM benzamidine, 1 mM α-aminocaproic acid, 1 mM phenylmethylsulfonyl fluoride). The suspended cells were broken by two passages through a French pressure cell (Aminco, 20,000 p.s.i.) at 0 to 4°C. Unbroken cells and large fragments of cell debris were removed by low speed centrifugation (6,000g, 10 min). Membranes were pelleted from the supernatant by high speed centrifugation (110,000g, 1 h), then suspended in buffer A containing sodium pyrophosphate (10 mM) and sodium bromide (1 M) to 40 ml. After incubation

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on ice for 40 min, the membranes were collected by high speed centrifugation (as above), then washed once in buffer A (using the same suspension and centrifugation steps), and finally were suspended in about 15 ml buffer A to yield a Chl concentration of 0.6 mg/ml. DβD-maltoside\(^3\) (Calbiochem-Behring) was added to the membrane suspension at a detergent to Chl ratio of 10:1 (w/w). After incubation on ice for 30 min, the preparation was centrifuged (110,000g, 1 h) and the supernatant, termed the maltoside extract, was collected. The maltoside treatment solubilized approximately 25 to 35% of the membrane Chl. Octylglucoside (Calbiochem) was then added to the maltoside extract to yield an octylglucoside to Chl ratio of 60:1 (w/w). The sample was incubated on ice for 30 min, then loaded onto linear sucrose density gradients (20 ml, 8–20% sucrose w/w; 2.5 ml of sample was loaded per gradient) containing buffer A and 0.03% (w/v) DβD-maltoside, and centrifuged for 3 h at 130,000g in a Ti 60 rotor. Two green bands (bands II and III) were in the middle and in the lower portion of the gradients, respectively. Material from a yellow band in the upper portion of the gradients (band I) was pooled and diluted with approximately four volumes of buffer A containing 0.03% (w/v) DβD-maltoside, was collected by cen-
trifugation (130,000g, 12 h), and then resuspended in a minimal volume of 50 mM Pipes (pH 7.5) containing 0.03% (w/v) DβD-maltoside. This sample was passed through a DEAE-Sephasel column equilibrated in the suspension buffer. Almost all yellow material eluted in the void volume. The yellow eluate was applied to an Affi-Gel 501 organomercury column (Bio-Rad Laboratories) equilibrated in the same buffer. The yellow eluate in the void volume from the Affi-Gel column was centrifuged at 130,000g for 24 h in the Ti 60 rotor, and the upper clear portion of the centrifuged solution was discarded. The yellow fraction, containing the carotenoid-associated protein, was collected and resuspended in a small volume of 50 mM Pipes (pH 7.5) containing 0.03% (w/v) DβD-maltoside. The properties of the carote-
noid on the columns and in ultracentrifugation suggest that the pigment was specifically bound to the protein.

Temperature-mediated phase partitioning of thylakoids was performed as described previously (4). The membranes used for these experiments were not washed with sodium pyrophosphate and sodium bromide solution.

Cytoplasmic membrane enriched fractions were obtained ac-

\footnote{Abbreviations: DβD-maltoside, dodecyl-β-D-maltoside; octylgluco-
side, octyl-β-D-glucopyranoside; LDS, lithium dodecyl sulfate; IPTG, isopropyl-β-D-thiogalactopyranoside.}

Other Measurements. PAGE in the presence of LDS was performed essentially as described in (12, 26). Samples for gel electrophoresis were solubilized at 0 or 70°C for 10 min. The marker proteins used as standards were BSA (67 kD), ovalbumin (45 kD), chymotrypsinogen A (25 kD), and Cyt c (12 kD). Proteins were detected by staining the gels with silver (35). Chl, phycocyanin, carotenoid, and protein concentrations were esti-

\[10000 \text{ spectrophotometer at } 25°C \text{ at a scanning speed of } 600 \text{ nm/min.
}\]

Temperature-difference spectra of yellow fractions suspended in 50 mM Pipes (pH 7.5), 0.03% (w/v) DβD-maltoside, 50% (v/v) glycerol were generated by subtracting a spectrum obtained at 25°C from a 0°C spectrum. Low temperature (77 K) fluorescence emission spectra were measured with an SLM 8000 spectrophluorometer (SLM Instruments Inc.).

RESULTS

Characteristics of Bands I and II Obtained from Sucrose Density Gradient. Yellow (band I) and green (bands II and III) bands were obtained from the sucrose density gradient. The red-

\[\text{region Chl absorption peak in normal } \text{Anacystis nidulans thyla-
koids is } 680 \text{ nm; the absorption peak in band II was } 676 \text{ nm,
}\]

whereas band I, where the content of Chl was considerably reduced, had a Chl absorption peak at 671 nm (Fig. 1A). Excita-
tion of \(A.~\text{niddulans} \text{thylakoids with } 435 \text{ nm light at } 77 \text{ K yiel-
d fluorescence emission peaks at } 685, 695, \text{ and } 716 \text{ nm (3); the}
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the 685 and 695 nm emission peaks are thought to arise from PSII-

\[\text{associated pigments, whereas the } 716 \text{ nm peak is from PSI-
associated Chl. Low temperature fluorescence emission spectra}
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from excited Chl in band II exhibited a large peak at 685 nm, a

\[\text{shoulder at } 695 \text{ nm, and very little fluorescence at } 716 \text{ nm,
}\]

indicating a significant enrichment of PSI relative to PSII in this

\[\text{fraction. By contrast, } 77 \text{ K fluorescence emission spectra of band}
\]

I Chl had a large peak at 685 nm and a shoulder at 673 nm (data

\[\text{not shown). Most of the PSI-associated Chl present in maltoside}
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extracts of thylakoids migrated with band III in the sucrose density gradient, and was not characterized further.

The major polypeptides present in band I and in band II are

\[\text{shown in Fig. 1B. The total maltoside extract material is enriched}
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in PSII-associated Chl-proteins at 52 and 45 kD (26). In band II

\[\text{material, these same two polypeptides are enriched still fur-
ther, as is a } 35 \text{ and a } 42 \text{ kD component (Fig. 1B). A more detailed}
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analysis of the PSIII-enriched band II material will be the subject

of a separate communication. Band I is depleted of the 52 kD

\[\text{PSII Chl-protein, but is slightly enriched in the } 45 \text{ kD Chl}
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protein and in the 71 kD phycoobilisome anchor component; the

most dramatic enrichment in band I, however, is in a 42 kD polypeptide (Fig. 1B, band I).

Carotenoid-Associated Protein Fraction. The yellow fraction

\[\text{obtained by passage of band I material through chromatography}
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columns was highly enriched in both carotenoids (free of Chl)

\[\text{and in the } 42 \text{ kD polypeptide (Fig. 2, A and B). The Synechocystis}
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PCC6714 carotenoprotein of cytoplasmic membranes changed
electrophoretic mobility from 35 to 45 kD when heated (6); an
immunologically related component displaying the same electrophoretic behavior exists in A. nidulans R2 (5). By contrast, the 42 kD polypeptide had the same mobility upon electrophoresis whether solubilized at 0°C or at 70°C (Fig. 2B). A trace amount of a 45 kD polypeptide is detectable in this yellow fraction upon solubilization at 70°C; however, on the basis of the relative enrichment of both the 42 kD polypeptide and carotenoids during the purification procedure, this contaminant is not likely to be responsible for the binding of a significant amount of carotenoid present in the purified fraction.

Temperature-mediated phase partitioning with Triton X-114 can separate the intrinsic membrane proteins from extrinsic components (4). Western blot experiments with the separated fractions of A. nidulans membranes obtained by Triton X-114 phase partitioning showed that only the intrinsic membrane protein phase contained the 42 kD polypeptide (data not shown). Hence, this carotenoid-containing protein appears to be different from the aqueous-phase carotenoprotein reported in Holt and Krogmann (14).

Cytoplasmic membrane preparations from A. nidulans R2 are enriched in a carotenoid species which absorbs light at 390 nm; this absorption maximum is much reduced in purified thylakoid membranes (24). The presence of such an absorption maximum in the isolated carotenoprotein would strongly suggest a cytoplasmic membrane origin for this species. The absorption spectrum of the isolated carotenoid-associated protein fraction is shown in Figure 2A. The major absorption peaks are at 460 and 487 nm, with a shoulder at 435 nm, and only a trace amount of Chl can be detected. Thus, this isolated carotenoprotein shows relatively little absorbance due to a carotenoid species having a peak at 390 nm. The isolated carotenoprotein and purified cytoplasmic membrane preparations both have major absorbance peak at 456 to 460 nm; the absorbance ratio (A390/A456) is therefore useful in assessing a possible contribution of cytoplasmic membrane carotenoids in these biochemical preparations. The A390/A456 ratios were 0.2 for this fraction, and 0.8 for cytoplasmic membranes isolated according to Omata and Murata (24). The relative absorption intensity at 390 nm in this preparation is therefore consistent with a thylakoid membrane origin of this carotenoprotein.

Omata and Murata (24) demonstrated that, in A. nidulans, cytoplasmic membrane-associated carotenoid absorption spectra

FIG. 1. Absorption spectra and polypeptide composition of bands I and II. Band I and band II were obtained by centrifugation of maltoside extracts on a sucrose density gradient. In A, the band preparations were suspended in buffer A prior to collection of absorption spectra at room temperature. B, polypeptide composition of band preparations (bands I and II). Polypeptides were analyzed by LDS-PAGE (13-18% acrylamide gradient) at 4°C, after solubilizing the samples of 0°C. The gels were stained with silver. The numerals at the right side in B show positions and mol wt (in kD) of marker proteins.
exhibited a marked spectral shift with a decrease in temperature, whereas thylakoid-associated carotenoids exhibited a much smaller shift. An absorbance-difference spectrum (0°C minus 28°C) of isolated cytoplasmic membranes shows a peak at 390 nm; the major carotenoid contributing to this peak seems to be zeaxanthin (11, 24). Cytoplasmic membrane enriched fractions obtained in our laboratory by the method of Omata and Murata (24) showed a similar spectrum (Fig. 3B). By contrast, our carotenoid fraction did not have such a peak in the difference spectrum (Fig. 3A), but yielded a reversible red-shift of carotenoid(s) (red-shift at 0°C). This result is similar to the difference spectrum obtained from the thylakoid fraction by Omata and Murata (24).

**Accumulation of the 42 kD Polypeptide under High-Light Growth Conditions.** *A. nidulans* cells grown under appropriate light conditions decrease their cellular content of PSI, whereas their phycobilisome and PSII content remain constant. This was shown to occur with cells grown in strong far-red light (23), light primarily absorbed by Chl a (9). *A. nidulans* cells grown under high white light intensities responded in much the same way as cells grown in strong far-red light. Although the phycocyanin to Chl ratios of cells grown in high and low light were similar, a blue shift of 2 nm in the Chl absorption band was present in phycobilisome-depleted thylakoids from cells grown in high-light, relative to identically treated thylakoids from cells grown in low light (678 nm and 680 nm, respectively). The 716 nm peak of 77 K Chl fluorescence emission of phycobilisome-depleted thylakoids from high-light cells was much less intense than that from low-light cells; additionally, the content of subunit I of PSI (12) in membranes from high-light cells was significantly less than that in low-light cells (standardized to constant membrane protein, data not shown). Western blotting experiments indicated that the quantity of the 42 kD protein present in the carotenoid-containing fraction (described earlier) increased dramatically in cells grown under high light conditions (Fig. 4A). In addition, there was a simultaneous increase in carotenoid content (about 150% on a Chl basis) in cells grown under high light. As a control, we monitored the levels of the extrinsic polypeptide associated with the oxygen-evolving PSII complex. This protein, isolated originally from chloroplasts by Kuwabara and Murata (19), has a mol wt of 33 kD in chloroplasts, but migrates as a 29 kD band in *A. nidulans* R2. This protein was chosen to determine if levels of PSII proteins were affected by changes in light quantity; as shown in Fig. 4A, this protein remained relatively constant during growth at either light intensity. This suggests that the increase in the 42 kD polypeptide is a response to high light, and is not specifically coupled to cellular PSII content.

Iron-stressed *A. nidulans* R2 cells decrease their thylakoid membrane content to 25 to 30% of normally grown cells, and decrease substantially the relative quantity of membrane proteins involved in photosynthesis (on a constant membrane protein basis; 29; HC Riehman, LA Sherman, unpublished data). If the carotenoprotein was in fact thylakoid-associated and involved in protection of photosynthetic proteins from photooxidative damage, we would expect it to respond to iron stress in the same way as the other photosynthetically important thylakoid proteins.

**Fig. 2.** Absorption spectrum and polypeptide composition of carotenoid-associated protein fraction. The absorption spectrum (A) was measured in a medium of 50 mM Pipes (pH 7.5) plus 0.03% (w/v) maltoside. Other conditions were the same as those in Figure 1. Samples for polypeptide analysis were solubilized at 0°C (B, left lane) or 70°C (B, right lane).
FIG. 3. Temperature-induced difference spectra (0°C minus 25°C) of the carotenoid-associated protein fraction and cytoplasmic membrane-enriched fraction. The cytoplasmic membrane-enriched fraction was obtained by the method of Omata and Murata (24). For measurement of the difference spectra, see "Materials and Methods."

Figure 4B is an immunoblot of electrophoretically separated components of *A. nidulans* membranes isolated from normal (+Fe) or iron-stressed (−Fe) cells. The 42 kD protein is detected only in normally grown cells, and is either absent or is much decreased in iron-stressed cells. This is consistent with both a photosynthesis-associated role and a thylakoid localization for this polypeptide.

The antibody generated against the purified carotenoprotein (Fig. 2B) was weakly reactive with several membrane components in addition to the strongly reactive 42 kD polypeptide, causing background bands to appear during immunostaining procedures (e.g. Fig. 4A, faint band at 35 kD). To eliminate background staining, we purified a fraction of the total antibody which was specific for a piece of the 42 kD polypeptide present on a β-galactosidase fusion protein ("Materials and Methods"). The purified antibody fraction was used to stain the immunoblot in Figure 4B. This purification of the antibody allowed us to confirm the identity of the 42 kD band as the major polypeptide stained in Figure 4A and, in addition, provides us with a highly specific reagent for further analysis of this protein.

**DISCUSSION**

A carotenoid-associated protein (42 kD) was isolated from DøD-maltosides extracts of *Anacystis nidulans* R2 membranes, and was enriched in the band I fraction from a sucrose density gradient (Fig. 1). This polypeptide is not a candidate for a cell wall-associated carotenoprotein for three reasons: (a) cell walls of *A. nidulans* have been reported to contain P750 (22), but band I had no such absorption peak (Fig. 1A); (b) the major polypeptides (50 and 52 kD) in the cell wall of *A. nidulans* (22, 27), are virtually absent in band I (Fig. 1B); and (c) the carotenoids of the cell wall fraction cannot be solubilized by Triton X-100 (18, 27), but the 42 kD polypeptide was easily solubilized by mild detergents.

The carotenoid composition of cytoplasmic membranes (where the major component is zeaxanthin) is known to be different from that in thylakoids (where the major carotenoid is β-carotene), and cytoplasmic membranes show a 390 nm peak in a temperature-induced difference spectra (11, 24). The isolated carotenoid-associated protein showed little contribution from

FIG. 4. Detection of immunoreactive components of membranes from cells grown under altered environmental conditions. Panel A, membranes isolated from cells grown in either low light (LL; 0.02 mW/cm²) or high light (HL; 1.9 mW/cm²) were fractionated using LDS-PAGE, and the separated components were electroblotted onto nitrocellulose filters. The left half of the LL lane was probed with antibody to the isolated 42 kD polypeptide, whereas the right half of the LL lane and the entire HL lane were probed with a mixture of antibody against the 42 kD polypeptide and antibody against the extrinsic 33 kD protein from spinach chloroplast oxygen-evolving particles. The membranes were solubilized at 0°C. The loaded amount of protein was 30 µg per lane. Panel B, membranes isolated from normal (+Fe) and iron-deficient (−Fe) cells were electrophoresed and blotted as in panel A, then probed with the affinity-purified antibody fraction described in "Materials and Methods." Detection of the primary antibody was with ¹²⁵I-conjugated goat anti-rabbit antibody, followed by autoradiography of the nitrocellulose filters.
this 390 nm absorbing carotenoid (Figs. 2 and 3), suggesting a different carotenoid composition from that in the cytoplasmic membrane. It is possible that the isolated carotenoprotein is associated with a subset of carotenoids and lipids of the cytoplasmic membrane whose behavior differs from that of the bulk cytoplasmic membrane material described in Omata and Murata (24); however, other data also argue for a thylakoid localization.

Membranes from iron-deficient cells contain a decreased quantity of many photosynthesis-related proteins; these include phycobilisome proteins, PSI subunits, cytochrome b5 complex subunits, and PSI reaction center proteins analogous to spinach CP47 and CP43 (HC Rietman, L. A. Sherman, unpublished data). By contrast, accumulation of a marker protein for the cytoplasmic membrane, the 35/45 kd polypeptide (5) is markedly enhanced in membranes from iron-deficient cells. These properties are probably due to both a general decrease in total thylakoid area in iron-stressed cells (29) and a more specific depletion of normal pigment-binding proteins with the concurrent accumulation of an iron stress-specific Chl-binding protein (26). The 42 kd polypeptide clearly follows the accumulation pattern of the photosynthesis-related thylakoid polypeptides with regard to iron stress (Fig. 4B), in contrast to the accumulation pattern of the cytoplasmic membrane marker protein. A 42 kd polypeptide is often found associated with the 71 kd phycobilisome 'anchoring' polypeptide in the A. nidulans R2 Chl-protein complex termed CP VI-1 (26, HC Rietman, L. A. Sherman, unpublished data). Isolated CP VI-1 material containing this 42 kd polypeptide was not reactive with antibody to the 42 kd carotenoprotein on Western blots (data not shown), ruling out one possible role for this carotenoprotein.

The isolated carotenoid-associated protein fraction did not show a spectral shift upon addition of tetraphenyllboron (up to 1 mM). A small pool of carotenoids in photosynthetic bacteria (16, 31, 34) and chloroplasts (16) responds to light with a small red shift of their absorption spectrum. This same pool of carotenoids is affected by the addition of tetraphenyllboron in the dark, but the result under these conditions is a small blue shift of the carotenoid absorption spectrum (16). Both effects are thought to be due to the perturbation of a permanent transmembrane electrical field in close proximity to the affected carotenoid molecules, which are assumed to be attached to light-harvesting pigment-protein complexes (16, 31, 34). Since addition of tetraphenyllboron to this A. nidulans carotenoid-associated protein fraction resulted in no detectable spectral shift (not shown), the carotenoid molecules in this fraction are not in an environment very different from the field-sensing carotenoids. This result is consistent with the findings of Itoh (16), who noted the absence of field-sensing pigments in A. nidulans thylakoids.

The carotenoid-associated protein isolated in this study is clearly different from the species isolated from the cytoplasmic membrane as described previously (5, 6). We have not been able to separate cleanly the cytoplasmic membrane from thylakoids in this strain of A. nidulans; hence, we present more indirect evidence which leads us to conclude that this carotenoid-binding protein is thylakoid-associated. More importantly, we have clearly shown that this carotenoid-associated protein does not accumulate in cells grown under low light conditions (0.02 mW/cm²), but becomes a fairly abundant membrane component in cells grown under both moderate (0.5 mW/cm²) and high (1.9 mW/cm²) light intensities. Although a specific role for this carotenoprotein has not yet been ascertained, the availability of the antibody will likely enable us to establish firmly the intracellular localization (through electron microscopy of immunogold-labeled cells) and possibly the in vivo function of this light-induced carotenoprotein (through selection, mutagenesis, and reintroduction of DNA clones encoding this protein).

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