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A zeaxanthin protein from *Anacystis nidulans*

Maria Diversé-Pierluissi and David W. Krogmann

Department of Biochemistry, Purdue University, West Lafayette, IN (U.S.A.)

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A carotenoid protein is present in an aqueous extract from the cyanobacterium *Anacystis nidulans*. The protein is easily purified to homogeneity and its absorption spectrum shows peaks at 430, 460 and 485 nm. The main carotenoid component is (3*R*, 3′*R*)-β,β-carotene-3,3′-diol (zeaxanthin) (83%) with small amounts of 13-*cis*-lutein (12%) and lutein (5%) present as well. The carotenoid protein complex is a dimer formed from two M_r 23000 peptides and approx. 44 carotenoid molecules.

Introduction

A water-soluble protein containing the carotenoid 3′-hydroxyechinenone has been found in three genera of cyanobacteria [1]. Here we describe the isolation and characterization of a similar but distinct carotenoid protein from another genus of cyanobacteria. This complex contains the dihydroxy carotenoid zeaxanthin and smaller amounts of 3′-*cis*-lutein, lutein and a larger polypeptide.

Most of the carotenoid pigments in cyanobacteria and in other photosynthetic organisms are embedded in the photosynthetic membranes. The carotenoid protein complexes described here and in Ref. 1 are unique in that they are soluble in aqueous media and can be purified without the use of detergents or organic solvents. Other carotenoid proteins can be released from the membranes of cyanobacteria by detergent extraction and must be purified in the presence of detergents. Sherman and Bullerjahn [2] purified and characterized a carotenoid protein from the cytoplasmic membranes of *Synechocystis* sp. strain PCC 6714. More recently Masumoto et al. [3] have isolated another carotenoid protein from the thylakoids of *Anacystis nidulans* R2. These proteins differ not only in their need for detergents to be released into and remain in aqueous solution but also in their spectral characteristics and in the molecular weights of the polypeptides.

In addition to the water-soluble and the detergent-solubilized carotenoid proteins, there is a third type of carotenoid protein in the light harvesting complexes and reaction center preparations where carotenoids are minor constituents often overshadowed by chlorophyll [4]. These carotenoid-chlorophyll protein complexes are intrinsic to the membrane and require stronger detergents for their solubilization.

Materials and Methods

A. nidulans (U. Tex 625) was obtained from the University of Texas culture collection and grown at 30 °C in Kratz and Myers medium C [5]. Cells were harvested by continuous flow centrifugation and resuspended in 0.1 M Tris-HCl buffer (pH 7.8) to a volume of 1 ml per g fresh weight of cells. Three cycles of freeze-thawing broke open the cells. Freshly harvested cells were also broken by

Correspondence: D.W. Krogmann, Department of Biochemistry, Purdue University, West Lafayette, IN 47907, U.S.A.

sonication with the same final result to establish that the carotenoid protein isolation was not dependent on freeze-thawing. The broken cell suspension was centrifuged at 8000 × *g* for 10 min to remove coarse debris, and the supernatant liquid was brought to 50% saturation with the addition of solid $(NH_4)_2SO_4$. After 24 h at 3° C, the precipitate was removed by centrifugation as above. The supernatant was brought to 95% saturation with $(NH_4)_2SO_4$ and, after 24 h, was centrifuged again. The protein precipitate was resuspended in a minimum volume of 5 mM Tris-HCl (pH 7.5) and dialysed against three 10-l volumes of this buffer for 24 h. The protein solution was centrifuged again as before and loaded onto a DEAE-52 ion exchange column (15 × 2 cm) which had been equilibrated with 5 mM Tris-HCl (pH 7.8). The column was washed with 50 ml of equilibrating buffer and was eluted stepwise using 50 ml of 0.05 M increments of NaCl up to 0.5 M. The carotenoid protein eluted from the column as a yellow band at 0.4 M NaCl. The protein was diluted 10-fold with distilled water and loaded on another DEAE column of the same size equilibrated with 5 mM phosphate buffer (pH 6.4). The yellow band eluted with 0.1 M NaCl. The protein was loaded on a Sephacryl S-200 (Pharmacia) gel filtration column equilibrated with 25 mM phosphate buffer (pH 6.4) and was eluted with the same buffer. 100 g of packed cells yield 2–4 mg pure protein.

Ultraviolet and visible absorption spectra of the protein were recorded on a Cary Model 14 spectrophotometer or an SLM-Aminco DW2 spectrophotometer. Absorption at single wavelengths was determined using a Zeiss PMQII model spectrophotometer.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according to Piccioni et al. [6] to determine protein molecular weight and purity. The molecular weight standards used were Pharmacia SDS-Molecular Weight Standards Kit, ranging from M_r 10000 to M_r 90000.

Molecular weight determination by gel filtration on Sephacryl S-200 was accomplished using a 2 × 90 cm column. The following molecular weight standards were used: phosphorylase *a* (100000) bovine serum albumin (67000), egg albumin (43000), chymotrypsinogen (25000) and horse heart cytochrome *c* (12500). The running buffer was 25 mM sodium phosphate (pH 6.4) with 0.10 M NaCl and the flow rate was 2.0 drops per min.

To remove the carotenoid from the protein, acetone was added to a concentrated solution of the carotenoid protein, denaturing the protein. The acetone was dried with a gentle stream of nitrogen, and acetone was added back to dissolve the carotenoid, the protein adhering to the vessel. The protein was yellow, showing that it still contained some carotenoid. A second extraction was done by adding butanol and storing for 48 h in the dark at 10° C; this removed most of the pigment but the pellet still had a faint yellow color.

Column chromatography was used for further purification of the pigment. A Brockmann Grade III alumina column [7] was packed in a Pasteur pipette. The pigment sample was dissolved in petroleum ether, loaded on a column and eluted with diethyl ether/petroleum ether mixtures of increasing diethyl ether content (0–100% diethyl ether). The pigment was eluted at 100% diethyl ether and only one visibly colored band was seen.

The next purification step was to load the sample onto a microcellulose column packed in a Pasteur column. A diatomaceous filter aid, Celite 545 (30–60% by weight) was added to improve the flow rate. The stepwise elution procedure, as used for alumina was used for this column. The pigment was eluted at 100% ethyl ether.

For the estimation of all carotenoids, the same extinction coefficient was used, $E^{1\%}_{1cm} = 2500$ in ethanol for λ_{max} [7].

For HPLC analysis of the carotenoid sample, a VYDAC TP201, 25 cm × 4.6 mm, 5 μm column and a mobile phase of methanol/chloroform (94 : 6) were used with a deuterium detector set at 475 nm. Spectral scans of the HPLC peaks were made by stopped flow method using a Beckman 165 variable wavelength detector [9].

The mass spectrum of the carotenoid was obtained on a Finnigan 4000 instrument with a Data General Nova/4 using a solid probe at 250° C and 70 eV.

Results

The absorption spectrum of the purified carotenoid protein is shown in Fig. 1. The peaks are at

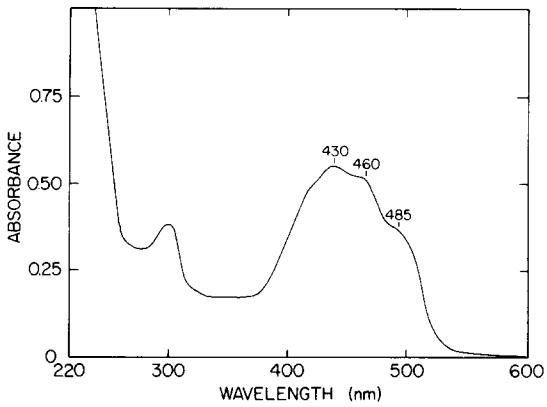

Fig. 1. Absorption spectrum of the carotenoid protein dissolved in 25 mM phosphate buffer (pH 6.4).

485, 460 and 430 nm. The A_{430}/A_{280} absorbance ratio was used used as a criterion of purity and in the best preparations was 1.6. This preparation showed a single protein band on SDS gel electrophoresis.

The minimum molecular weight of the polypeptide in the carotenoid protein was determined by SDS-polyacrylamide gel electrophoresis. The detergent removed the carotenoid from the polypeptide. A single band corresponding to a molecular weight of 23 000 was obtained. The molecular weight of the intact carotenoid protein complex was estimated by gel filtration chromatography on a Sephacryl S-200 column. The complex elutes in a volume which corresponds to a molecular weight of 71 500.

Fig. 2 shows the absorption spectra in acetone and in hexane of the carotenoid pigments removed

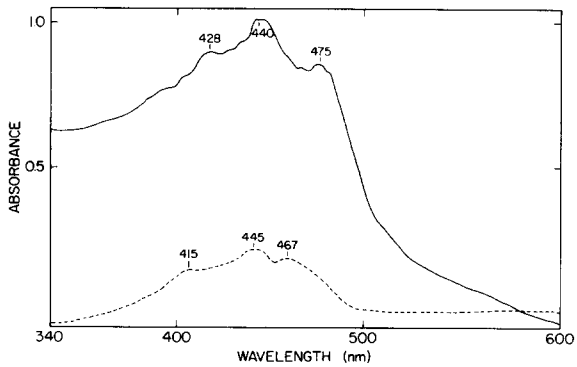

Fig. 2. Absorption spectra of the carotenoid in hexane (solid line) and in acetone (dashed line).

TABLE I

HPLC DATA OF ACETONE-EXTRACTED CAROTENOIDS

Peak number	1st Acetone extract (area %)	2nd Butanol extract (area %)	Retention time	
1	1.052	0.927	3.0	solvent interface
2	11.726	16.357	4.90	13-*cis*-lutein
3	3.787	13.452	5.81	lutein
4	83.435	60.828	6.45	zeaxanthin

from the purified protein by acetone extraction. These spectra suggest a C_{40} carotenoid with two closed beta ionone rings. The behavior of the pigment on alumina and on microcellulose columns indicated hydroxylation and the stepwise elution which released the pigment only at 100% diethyl ether indicated a dihydroxy- or a trihydroxycarotenoid.

The carotenoid pigment was resolved into three distinct bands by chromatographing on a VYDAC TP201 HPLC column and the peaks could be identified by comparison to the behavior of standard carotenoids on this column. Table I shows the retention time of each peak and its identity. Comparison of these retention times with the mean retention times of standards run under these conditions indicates that the 4.9 peak corresponds to 13-*cis*-lutein, the 5.81 peak to lutein and the 6.45

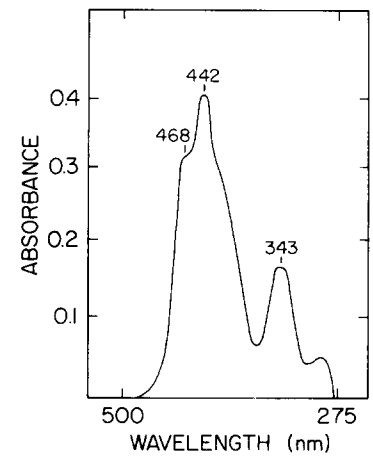

Fig. 3. Absorption spectrum of 13-*cis*-lutein on elution from an HPLC column.

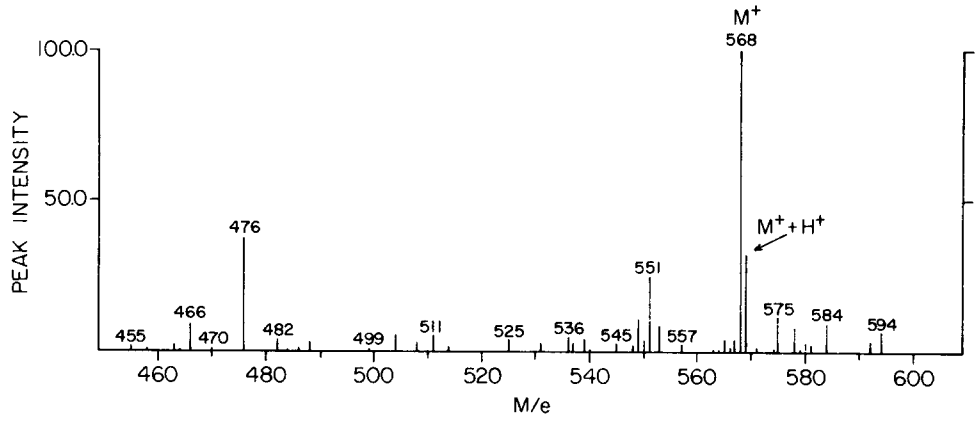

Fig. 4. Mass spectrum of the carotenoid extracted from the carotenoid protein complex.

peak to zeaxanthin. The identification of the 13-*cis*-lutein is confirmed by its distinctive absorption spectrum in the HPLC mobile phase shown in Fig. 3. The peak at 343 nm indicates the presence of a *cis* double bond and the ratio of the absorbance at 442 nm to 343 nm of Fig. 3 indicates that it is 13-*cis*.

The identity of the zeaxanthin peak was confirmed by mass spectrometry. Fig. 4 shows the upper end of the mass spectrum of the dominant carotenoid in a chemical impact analysis. The parent ion peak at M/e 568 is the molecular weight of zeaxanthin. The large peak at M/e 476 is characteristic of carotenoids and is ascribed to elimination of part of the polyene chain [10]. The peak at M/e 551 indicates the loss of one water molecule $[(M+H^+)-18]$ which is expected of the alcohol groups on the zeaxanthin diol. This experimental mass spectrum matches the spectrum for zeaxanthin published by the EPA/NIH Mass Spectral Data Base.

Fig. 5 shows the structures of the three carotenoids on the purified carotenoid protein from *A. nidulans*. In addition, the structure of 3'-hydroxyechinenone is shown. This carotenoid was found on a similar protein isolated from *Microcystis*

TABLE II

ANACYSTIS NIDULANS CAROTENOID PROTEIN

Polypeptide monomer without carotenoids (SDS gel)	23000
Two copies of above	46000
44 carotenoids (44×568)	24500
Carotenoid protein complex (gel filtration column)	71500

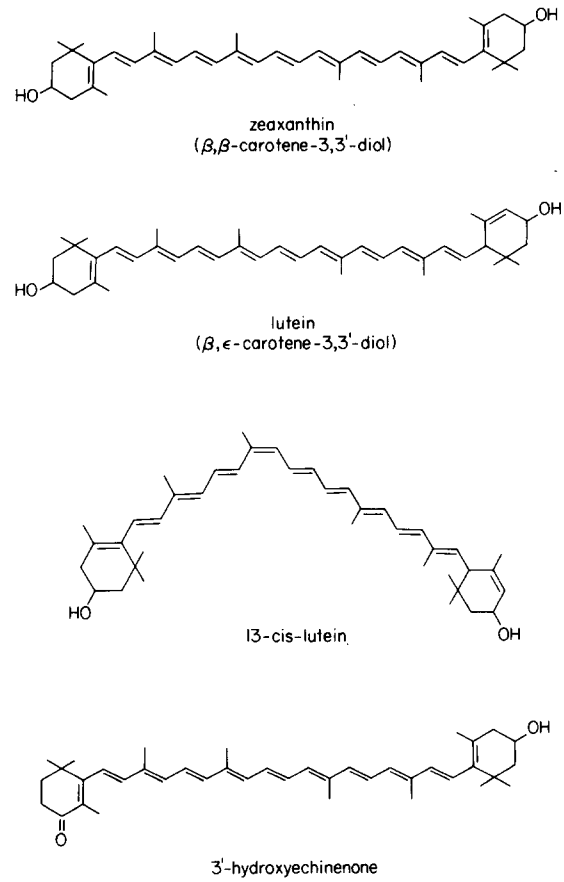

Fig. 5. Structures of carotenoids in carotenoid protein complexes.

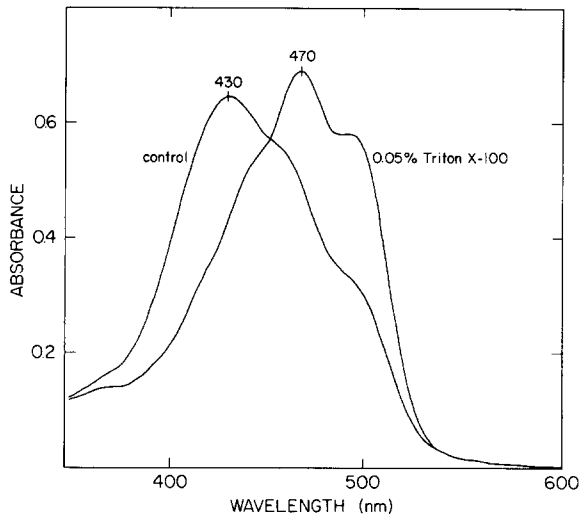

Fig. 6. Absorption spectrum of the carotenoid before and after the addition of Triton X-100.

aueruginosa, Spirullina maxima and *Aphanizomenon flos-aquae* [1].

Table II contains data from measurements on several preparations in which the amount of carotenoid estimated in organic solvent extracts is related to the amount of protein estimated from ultraviolet absorbance and Biuret determination. Carefully weighed samples of purified protein were extracted with organic solvents to give the contribution of carotenoid, as determined spectroscopically, to the total mass. When the residual mass was divided by the molecular weight of the polypeptide as determined by SDS-polyacrylamide gel electrophoresis one could calculate the same stoichiometry of pigment to protein. The table summarizes the contributions of each constituent to the mass of the complex.

Fig. 6 provides a cautionary note to the study of carotenoid proteins. The visible absorption spectrum of the carotenoid protein complex is altered by the presence of the detergent Triton X-100.

Discussion

Water-soluble carotenoid proteins seem to be frequent but not universal constituents of cyanobacteria. This paper establishes their occurrence in four genera but we have failed to observe them in similar fractionation experiments with extracts of many other cyanobacteria. This absence may be due to growth conditions or internal regulation which prevented the synthesis of the carotenoid protein. The function of these proteins is unknown, as is their intracellular location – either dissolved in the cytosol or loosely attached to a membrane. The constant ratio of visible to ultraviolet light absorption seen in many preparations purified to homogeneity indicates a fixed stoichiometry between carotenoid and protein. The carotenoid protein of *A. nidulans* has more carotenoid (44 vs. 36 pigment molecules) and a larger polypeptide subunit (23 000 vs 16 000) than the 3′-hydroxyechinenone protein of *M. aeruginosa, S. maxima* and *A. flos-aquae*. The *A. nidulans* zeaxanthin protein is very much more stable than the other which suffered a rapid and spontaneous color change and dissociation to monomeric units.

The binding of zeaxanthin to the polypeptide causes a slight distortion of the chromophore absorption (the peak positions on the protein complex are 430, 460, and 485 nm compared to those of the pigments dissolved in hexane (428, 440, 475 nm) or in acetone (415, 445 and 467 nm)). This may indicate that the carotenoids are held close to one another rather than spread thinly over the polypeptide. The release of relatively more 13-*cis*-lutein and lutein in the second extraction with butanol suggests that these pigments are bound more tightly than zeaxanthin to the polypeptide.

In earlier work on the 3′-hydroxyechinenone protein, we had noticed that the absorption spectrum of the complex was distorted by exposure to any of a large number of detergents. The zeaxanthin protein responds in a similar fashion. Fortunately, no detergent is needed in the isolation of these proteins so they may be a more convenient object for study of chromophore-protein interactions.

Murata et al. [11] found that zeaxanthin is the most abundant carotene in *A. nidulans* and is especially prominent as 78% of the carotene in the cell envelope membrane. The zeaxanthin in the carotenoid protein described in work reported here can be no more than a few percent of the total zeaxanthin in these cells.

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