15-*cis*-β-Carotene found in the reaction center of spinach photosystem II

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Abstract Solvent extraction at ~4°C in complete darkness, and subsequent analysis by high-pressure liquid chromatography (at ~4°C) using an apparatus equipped with a two-dimensional diode-array detector, spectroscopically identified 15-*cis*-β-carotene in the reaction center (RC) of spinach photosystem II (PS II). The result revises a previous conclusion that β-carotene bound to the PS II RC takes the all-trans configuration [Fujiwara, H., Hayashi, H., Tasumi, M., Kanaji, M., Koyama, Y. and Satoh, K. (1987) Chem. Lett. 2005–2008], and generalizes the concept established for purple photosynthetic bacteria that 15-*cis*-carotenoid is naturally selected by the photosynthetic reaction centers.

Key words: Photosynthesis; β-Carotene; Reaction center; Photosystem II;15-*cis* Configuration; Spinach

1. Introduction

Carotenoids in photosynthetic systems have two different functions, i.e. light-harvesting and photo-protection [1–4]. In relation to these two functions, the natural selection of the carotenoid configurations has been found in the pigment-protein complexes of purple photosynthetic bacteria (reviewed in [3]): the *all-trans* configuration is selected by the light-harvesting complexes (LHC) where the light-harvesting function is expected to predominate. On the other hand, the 15-*cis* configuration is selected by the reaction center (RC) where the photoprotective function is most important. dHere, the 15-*cis* configuration must have advantage over all the other *cis–trans* configurations in pursuing the photo-protective function, i.e. quenching triplet bacteriochlorophyll and dissipating the triplet energy to the surroundings as heat.

The RCs in photosynthetic organisms are conventionally classified into two types, i.e. 'quinone type' and 'ion-sulfur type'. The 'quinone type' RC is used both in purple bacteria and in photosystem II (PS II) of oxygenic photosynthetic organisms [5]. Since strong homology has been found between the PS II RC and the RCs of purple photosynthetic bacteria in the composition of the prosthetic groups for the electron-transfer reactions as well as in the amino acid sequences of the apo-peptides [6], an obvious question to be addressed is: 'does β-carotene in the PS II RC also take a 15-*cis* configuration?'. A first attempt to identify 15-*cis*-β-carotene in the PS II RC was unsuccessful [7], and it was concluded that β-carotene takes the *all-trans* configuration in this system.

However, the 15-*cis* isomer of β-carotene is extremely unstable when excited to the triplet state, and it rapidly isomerizes into *all-trans* (reviewed in [8]): time-resolved Raman and electronic absorption spectroscopies could identify not the '15-*cis*' T_1 but the '*all-trans*' T_1, a product of the T_1-state isomerization, and analysis by high-pressure liquid chromatography (HPLC) of triplet-sensitized isomerization determined the quantum yield of the 15-*cis* to *all-trans* isomerization to be almost unity. Since chlorophyll *a*, a strong triplet sensitizer, is always present in the PS II RC, there was a good chance that 15-*cis*-β-carotene intrinsically bound to the pigment protein complex isomerized during the processes of extraction and of HPLC analysis. Thermal isomerization from 15-*cis* to *all-trans* has also been found. Recently, we have developed a technique to isolate the 15-*cis* isomers of neurosporene, spirilloxanthin (see [3]), and spheroidene from the RCs of *Rhodobacter sphaeroides* G1C, *Rhodospirillum rubrum* S1 and *R. sphaeroides* 2.4.1, but this technique, as it was, did not apply successfully to the case of β-carotene in the PS II RC. In the present investigation, we have attempted to identify 15-*cis*-β-carotene by a combination of: (i) extraction of the carotenoid component at ~4°C in complete darkness; and (ii) analysis by HPLC of the extract at ~4°C using a two-dimensional diode-array detector which facilitates simultaneous electronic-absorption measurements.

2. Materials and methods

2.1. Preparation of the PS II RC

The PS II RC (the D1-D2 Cyt *b*-559 complex) was isolated from spinach grana thylakoids by a method adopted from that described previously [9]: the PS II particles [10] (1 mg of chlorophyll per ml) were treated with 4% (w/v) Triton X-100 in 50 mM Tris-HCl (pH 7.2) for 2 h at 4°C with stirring. The resultant mixture was centrifuged at 100,000 × *g* for 1 h at 4°C, and the supernatant solution was adsorbed onto a DEAE-Toyopearl 650S column equilibrated with a buffer solution consisting of 50 mM Tris-HCl (pH 7.2), 0.05% Triton X-100, and 30 mM NaCl. The column was then washed with the same buffer solution until the eluent became colorless. The column was then subjected to the gradient elution of NaCl (60–120 mM) in the presence of 0.05% Triton X-100 and 50 mM Tris-HCl (pH 7.2), and the major green fraction was collected as the RC component. The detergent and the buffer were subsequently replaced by 0.15% sucrose monocaprate and 50 mM HEPES-NaOH (pH 7.5), respectively. Before extraction, the PS II RC was incubated at 0 °C in the dark for 2 h.

2.2. Extraction and HPLC analysis

β-Carotene was extracted from the PS II RC as follows: 2 ml of *N,N*-dimethylformamide (Kishida Chemicals, special grade), 1 ml of *n*-hexane (Kishida Chemicals, special grade), and then 2 ml of distilled water, were added in this sequence to 1 ml of the RC (OD at 676 nm, 17.5 cm^{-1}) dissolved in 0.15% sucrose monocaprate (Dojin Chemicals, special grade) and 50 mM HEPES-NaOH (Dojin Chemicals, special grade) at pH 7.5. (In some extractions, acetone (Kishida chemicals, special grade) was used instead of *N,N*-dimethylformamide.) After each solvent addition, the mixture was vibrated for 2 s and then centrifuged for 10 s at 3500 rpm. The hexane layer with pigments was passed

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through an alumina (Merck, activity II-III, 63–200 μm) column (5 mm (i.d.) × 15 mm), and then it was subjected to HPLC analysis. The HPLC conditions [11,12] were as follows: column, 4 mm (i.d.) × 250 mm packed at 300 kg/cm^2 with calcium hydroxide (Nacalai Tesque, special grade; lot # M6G2126); eluent, 0.1% acetone in *n*-hexane; flow rate, 0.5 ml·min^{-1}; and detection, 450 nm. A Shimadzu LC-10AS liquid chromatograph equipped with a Waters 996 photodiode-array detector and a Millennium 2010 software were used for acquisition and analysis of the data. (See below for precautions to prevent isomerization.)

3. Results and discussion

We have taken the following precautions to avoid isomerization of the extremely unstable 15-*cis*-β-carotene: In order to prevent triplet-sensitized photo-isomerization: (1) all the procedures of extraction and HPLC analysis were done in complete darkness, except for injection of the extracted carotenoid to the HPLC apparatus in the dim red light (the injection ought to be quick, but not to be turbulent.); and (2) chlorophyll *a* was removed by passing the extracted pigments in *n*-hexane layer through a very short alumina column. To avoid thermal isomerization: (3) the suspension of the PS II RC was incubated at 0°C in complete darkness for 2 h before extraction; (4) all the solvents (eluents), containers, test tubes, pipettes, and syringes which were necessary for the extraction and HPLC analysis were cooled on ice; (5) the liquid lines (stainless-steel and teflon tubings) were cut to a minimum length, and the HPLC column and the liquid line were thermally insulated; and (6) the room temperature was set at 18°C, and the entire HPLC system was stabilized by running the ice-cooled eluent for two days before starting the experiment. To facilitate efficient acquisition of electronic absorption data; (7) a two-dimensional, photodiode-array detector system was introduced, so that the electronic absorption spectrum of each peak could be determined during the HPLC analysis; and (8) *N,N*-dimethylformamide was used as a solvent for extraction to identify a *cis* isomer by the intensity of the *cis*-peak (the $^1A_g^+ \leftarrow {}^1A_g^-$ transition) of the carotenoid. Acetone, instead, was used as a more efficient solvent to avoid isomerization. However, the absorption of acetone remaining in *n*-hexane layer prevented the configurational identification by the *cis*-peak. Retention times of the isomers were changed depending on the solvent used for extraction.

Fig. 1 shows a three-dimensional chromatogram in the HPLC analysis of β-carotene for the *N,N*-dimethylformamide extract. Clearly seen are two components, i.e. component 1 eluting around 9 min, and component 2 eluting around 12.5 min. There is an additional minor component eluting around 10 min. It can be assigned to an intermediate in the 15-*cis* to *all-trans* isomerization; this component disappears when the

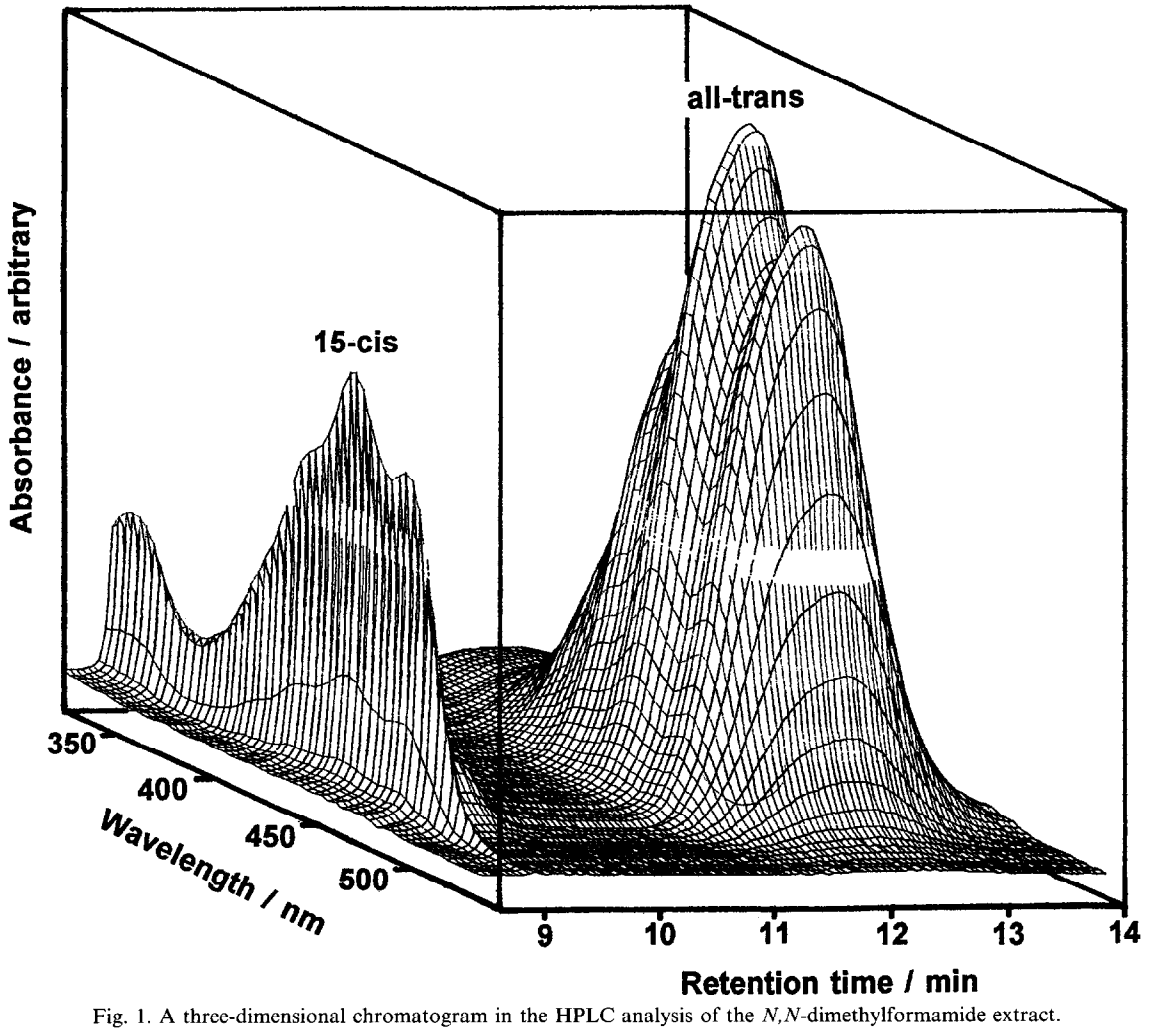

Fig. 1. A three-dimensional chromatogram in the HPLC analysis of the *N,N*-dimethylformamide extract.

15-*cis* to *all-trans* isomerization is completed. Fig. 2 compares (a) the absorption spectrum of component 1 with (b) that of synthetic 15-*cis*-β-carotene purified by HPLC [11,12]. Almost complete spectral agreement establishes that component 1 in the extract is actually the 15-*cis* isomer of β-carotene, i.e. the maximum difference in the wavelengths of the three peaks is 0.6 nm for the spectral resolution of 1.2 nm. The relative intensity of the *cis*-peak at 337 nm looks slightly lower than that of synthetic 15-*cis*-β-carotene, a fact which suggests the 15-*cis* to *all-trans* isomerization taking place even in the tubings of the HPLC apparatus. The possibility that component 1 is another *cis* isomer of β-carotene can be excluded completely when the wavelength of the 0–0 peak of the $^1B_u^+ \leftarrow {}^1A_g^-$ transition of component 1 (475 nm) is compared with those of a set of *cis–trans* isomers of β-carotene [13]. Fig. 2 also compares (c) the absorption spectrum of component 2 with (d) that of synthetic *all-trans*-β-carotene. The spectral agreement shows that component 2 is the *all-trans* isomer of β-carotene. The maximum difference in the $^1B_u^+ \leftarrow {}^1A_g^-$ vibrational structures is only 0.4 nm, and no *cis* peak is seen in the spectra. Fig. 3 depicts the structures of the (a) 15-*cis* and (b) *all-trans* isomers of β-carotene.

Fig. 4 shows an HPLC elution profile of β-carotene for the acetone extract. The peak eluting around 7 min can be assigned to component 1 (15-*cis*-β-carotene), while the peak eluting around 16 min can be assigned to component 2 (*all-trans*-β-carotene); both assignments are based on the wavelengths of the $^1B_u^+ \leftarrow {}^1A_g^-$ vibrational structures [13]. (The plateau between the pair of peaks suggest that the 15-*cis* to *all-trans* isomerization is taking place during the adsorption/desorption processes in the calcium hydroxide column.) The peak heights of component 1 and component 2 are in the ratio of 95:4. When the peak width is assumed to be the same, and when the relative molar extinction coefficient of *all-trans*/15-*cis* = 1.36 at 450 nm is taken into account, the molar ratio of 15-*cis*/*all-trans* can then be estimated to be 96:4. The result strongly suggests that the two β-carotene molecules in the PS II RC [14] take the 15-*cis* configuration, although the presence of functionally different β-carotene molecules has been suggested in this pigment–protein complex [15]. Thus, a positive answer to the question which has been addressed in section 2 has been obtained.

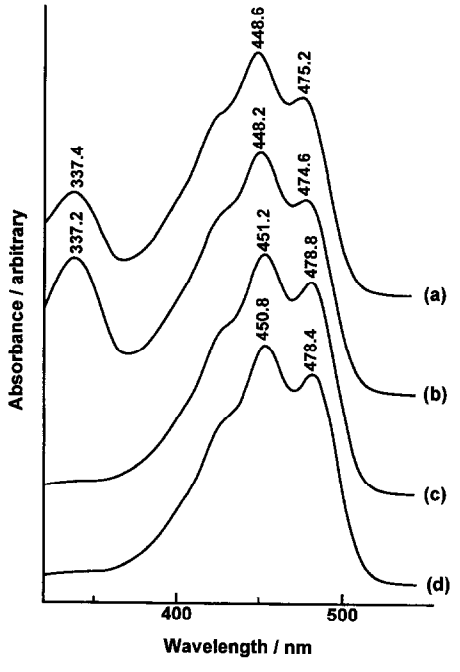

Fig. 2. Comparison of the electronic absorption spectra in *n*-hexane between the components in the extracted β-carotene from spinach PS II RC complex and the authentic isomeric β-carotenes. (a) Component 1, (b) synthetic 15-*cis*-β-carotene, (c) component 2, and (d) synthetic *all-trans*-β-carotene. The ordinate scales of the original spectra were: (a) 0.043, (b) 0.95, (c) 0.113 and (d) 0.133 absorbance unit at the absorption maxima.

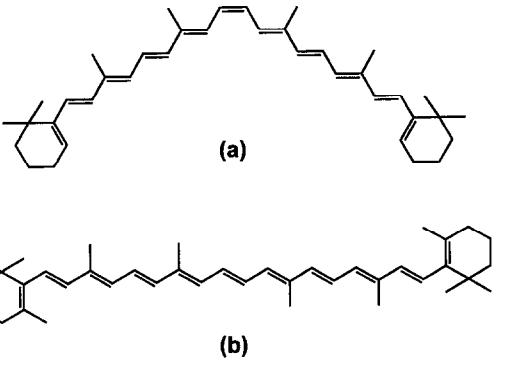

Fig. 3. Structures of (a) 15-*cis*- and (b) *all-trans*-β-carotenes.

The present results revise the previous conclusion that β-carotene bound to the PS II RC takes the *all-trans* configuration [7]. Test experiments using experimental conditions similar to those in the previous investigation gave rise to 'pure' *all-trans* isomer (in other words, the 15-*cis* to *all-trans* isomerization was completed). Actually, all the modifications from the extraction and HPLC conditions which are described in this paper showed that the 15-*cis* to *all-trans* isomerization can take place at every step. Changes in the electronic absorption pattern depending on the retention time showed that the above isomerization can take place in the column and in the tubings. The 15-*cis* to *all-trans* isomerization most probably originates from a trace of the strong sensitizer, chlorophyll *a*, which absorbs infrared light.

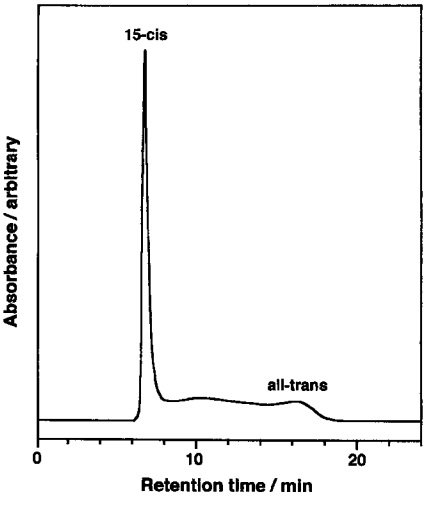

Fig. 4. An HPLC elution profile for the acetone extract (detection at 450 nm).

In the previous investigation, the 15-*cis* configuration could not be detected even by Raman spectroscopy [7]. This may be ascribable to triplet-sensitized isomerization within the pigment–protein complex [16]; however, further investigation is necessary to establish this interpretation.

The present finding of the 15-*cis* isomer in the PS II RC, where the photo-protective function is most crucial due to the presence of the oxygen-evolving system, strongly suggests a universal role of the 15-*cis* configuration for the photo-protective function, i.e. accepting and then dissipating the triplet energy.

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