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The presence of 9-*cis*-β-carotene in cytochrome b_6f complex from spinach

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Abstract

Cytochrome b_6f complex with stoichiometrically bound β-carotene molecule was purified from spinach chloroplasts. The configuration of this β-carotene was studied by reversed-phase HPLC and resonance Raman spectroscopy. Both the absorption spectrum of this β-carotene in dissociated state and the Raman spectrum in native state can be unambiguously assigned to a 9-*cis* configuration. This finding is in contrast to the predominantly all-*trans* isomers commonly found in membranes and protein–pigment complexes of chloroplasts, suggesting that the 9-*cis*-β-carotene is an authentic component and may have a unique structural and functional role in cytochrome b_6f complex. © 2001 Published by Elsevier Science B.V.

Keywords: Cytochrome b_6f; β-Carotene; Configuration; High performance liquid chromatography; Resonance Raman spectroscopy

1. Introduction

The cytochrome (Cyt) b_6f complex is an integral membrane protein complex in thylakoid active as a plastoquinol-plastocyanin oxidoreductase, mediates electron transfer between two reaction center complexes and also translocates protons across the membrane. It is composed of four large (18–32 kDa) subunits binding one *c*-type heme (Cyt *f*), two *b*-type hemes (Cyt b_6), one high potential iron–sulfur center (Rieske iron–sulfur protein), plastoquinone, -quinol, or -semiquinone (subunit IV and Cyt b_6), and four small subunits (<5 kDa) without prosthetic groups [1,2]. Approximately one chlorophyll (Chl) *a* molecule and one or less than one carotenoid molecule per monomer have been found in isolated Cyt b_6f complexes [3–7,14], which were previously thought to be contaminants [8,9]. Recent research has provided evidence that this chlorophyll *a* molecule is an authentic component with a specific binding site in Cyt b_6f complex, in spite of the enigma of its functional role in this complex [10–13]. Relatively few studies have been done on the carotenoid in Cyt b_6f complex. It is interesting to note that echinenone, which was detected only in Cyt b_6 after all subunits were dissociated, is the only carotenoid bound to the Cyt b_6f complex from the cyanobacterium *Synechocystis* PCC6803, although β-carotene is more abundant in *Synechocystis* [14]. A photoprotective function for the carotenoid was inferred from observations that the rate of photobleaching of the Chl *a* bound in the Cyt b_6f complexes from different organisms was found to vary inversely with β-carotene content and to decrease markedly in the presence of ambient N_2 instead of air [7]. These obser-

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vations suggest that the carotene in the isolated Cyt b_6f complexes is not a contaminant but could be an important structural/functional component.

The carotenoid in spinach Cyt b_6f complex was identified to be β-carotene by mass spectroscopy and its configuration was speculated to be 9-*cis* or 15-*cis* from its absorption spectrum [7]. However, it is recently reported [15] that Raman bands (441.6 nm excitation) from this β-carotene can be assigned to an all-*trans* configuration. In this work, reversed-phase HPLC and resonance (488 nm excitation) Raman spectroscopy were used to determine the configuration of this β-carotene. A 9-*cis* configuration in both the dissociated and native states was revealed from the absorption and the resonance Raman spectra of the β-carotene in Cyt b_6f complex from spinach. In contrast to the all-*trans* configuration commonly observed for β-carotene in membrane and pigment–proteins of chloroplasts, this 9-*cis* configuration provides additional evidence that β-carotene is an authentic component playing an important structural/functional role in the Cyt b_6f complex.

2. Materials and methods

2.1. Materials

n-Octyl-β-D-glucopyranoside (β-OG), phenylmethylsulfonyl fluoride (PMSF), benzamidine, and ε-aminocaproic acid, sodium cholate, chlorophyll *a* and all-*trans*-β-carotene standards were purchased from Sigma. 9-*cis*-β-Carotene standard was a kind gift from Prof. Yasushi Koyama, Kwansei Gakuin University, Japan.

2.2. Purification of cytochrome b_6f complex

Cytochrome b_6f complex was purified at 4°C under dim light employing two ammonium sulfate fractionation processes as described previously [5,6]. Briefly, chloroplasts were isolated from prechilled fresh spinach leaves, and then washed with 0.15 M NaCl, osmotically broken with 10 mM Tricine–NaOH (pH 8.0), washed again with 2 M NaBr followed by 0.15 M NaCl as in Doyle and Yu [9]. For selective extraction of Cyt b_6f complex, the thylakoid membranes were resuspended in 20 mM Tricine–NaOH (pH 8.0), 0.2 M sucrose, 3 mM $MgCl_2$, 3 mM KCl, 40 μM PMSF, 1 mM benzamidine, and 2 mM ε-aminocaproic acid containing 30 mM β-OG and 0.5% sodium cholate at a chlorophyll concentration of 1.5 g/l, and incubated for 30 min with continuous stirring. The suspension was centrifuged at $30\,000\times g$ for 30 min. The supernatant was fractionated by ammonium sulfate precipitation. Crude Cyt b_6f complex precipitated from 55–60% saturation of ammonium sulfate was collected and resuspended in 5 ml 30 mM Tris–succinate (pH 7.0), 3 mM $MgCl_2$, 3 mM KCl, 40 μM PMSF, 1 mM benzamidine, and 2 mM ε-aminocaproic acid containing 0.5% sodium cholate, and dialyzed for ∼12 h against 30 mM Tris–succinate (pH 7.0), 25 mM sucrose, 10 mM NaCl and 40 μM PMSF. The dialyzed protein solution was diluted with an equal volume of prechilled water, left standing for 60 min, and then centrifuged at $40\,000\times g$ for 50 min. The Cyt b_6f pellet was resuspended to a Cyt *f* concentration of ∼15 μM in 20 mM Tricine–NaOH (pH 8.0), 0.2 M sucrose, 40 μM PMSF, 1 mM benzamidine, and 2 mM ε-aminocaproic acid containing 30 mM β-OG and 0.5% sodium cholate, stirred for 20 min and then centrifuged at $10\,000\times g$ for 6 min. The supernatant was then fractionated by ammonium sulfate precipitation again. Pure Cyt b_6f was precipitated from 43–56% saturation of ammonium sulfate. The precipitated Cyt b_6f was resuspended in 50 mM Tricine–NaOH (pH 8.0) containing 30 mM β-OG, and stored at −80°C. Reduced minus oxidized extinction coefficient, $\Delta\varepsilon$ (554–540 nm) = 28 mM^{-1} cm^{-1}, of Cyt *f* [16] was used to determine cytochrome b_6f concentration.

2.3. Reversed-phase HPLC analysis of the β-carotene extracted from cytochrome b_6f complex

Pigments were extracted with 80% acetone at 4°C under dim light, sonicated for 2 min, centrifuged at $5000\times g$ for 5 min, and the supernatant was collected. To ensure complete extraction, the residual pigments in the precipitate were extracted with the above procedure for one more time, and then the supernatants were combined for HPLC analysis. HPLC analysis was carried out with a Waters 600 liquid chromatograph equipped with a Waters 996 photodiode array detector, and a Discovery C18 reversed-phase column (Supelco, 25 cm×4.6 mm,

5 μm granulometry). The column was equilibrated with methanol for 10 min. The sample was eluted by 1 min linear gradient to methanol:hexane (4:1, v/v, mixed before use), and then eluted isocratically (17 min) with methanol:hexane (4:1, v/v). Sample injection was 20 μl, the flow rate was 1.0 ml/min. The absorption spectra of the eluted fractions were continuously monitored with a Waters 996 photodiode array detector (wavelength range 300–750 nm). The HPLC chromatogram and corresponding absorption spectra of the resolved components of β-carotene were obtained with the Waters Millennium 2010 software.

2.4. Resonance Raman spectroscopy

Resonance Raman spectra were obtained with a Jobin Yvon T8000 Raman spectrometer. About 35 μM Cyt b_6f suspended in 50 mM Tricine–NaOH, pH 8.0, 30 mM β-OG was used for Raman spectrum measurement. The 488 nm excitation (less than 10 mW on the sample) was provided by an Argon laser. To prevent photodamage of the pigments, the sample was cooled at 77 K in a capillary by liquid nitrogen during spectral measurement.

3. Results

3.1. Characterization of purified spinach cytochrome b_6f complex

Fig. 1 shows the polypeptide pattern of the purified Cyt b_6f complex. Only four polypeptides corresponding to the four major subunits of Cyt b_6f complex were resolved in the pattern of SDS–PAGE. The activity of the Cyt b_6f complex used in this work was ~120 electrons transferred from decyl-plastoquinol to plastocyanin-ferricyanide per Cyt f per second, in agreement with previous measurements [3,7].

The composition of the pigments in the Cyt b_6f complex was analyzed by HPLC. Fig. 2 displays the HPLC chromatogram of the pigments extracted from spinach Cyt b_6f complex. One major and one minor β-carotene isomer, and one chlorophyll a component were resolved by reverse phase HPLC in methanol:hexane (4:1) as detected by their absorbance at 440 nm. The concentration of each pigment

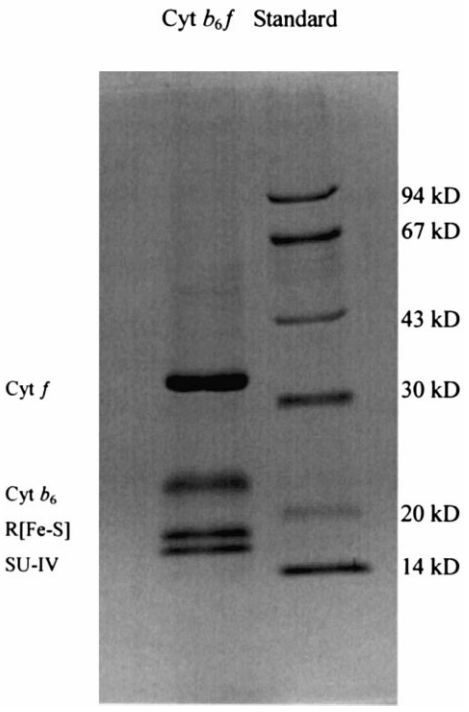

Fig. 1. SDS–PAGE (linear gradient slab gels from 10–15% polyacrylamide) of the purified Cyt b_6f complex from spinach chloroplasts.

component separated by HPLC was determined by comparing areas under the peak in the chromatogram with that of the known concentration of chlorophyll a or β-carotene standard. The extinction coefficients of $\Delta\varepsilon$ (A_{665}–A_{670}) = 71.4 mM^{-1} cm^{-1} for chlorophyll a in methanol [17] and ε (445 nm) = 134 mM^{-1} cm^{-1} for β-carotene in hexane [18] were used to determine the concentration of pigment standards. The molecular ratios of the chlorophyll a, the major β-carotene and the minor β-carotene components to Cyt f in this b_6f complex were determined to be 1.52, 0.84 and 0.15, respectively.

3.2. Absorption spectra of β-carotene extracted from cytochrome b_6f complex

It has been shown that the position of the absorbance maximum at the longest wavelength and the relative intensity of the peak around 340 nm ('*cis*-peak') are sensitive to the configuration of each β-carotene isomer [19,20]. Fig. 3 shows the absorption spectra of the β-carotene extracted from spinach Cyt b_6f, 9-*cis*- and all-*trans*-β-carotene standards resolved

by reversed-phase HPLC. The spectrum of the minor β-carotene component shows two intense peaks around 451 and 478 nm, consistent with that of the all-*trans*-β-carotene standard. The spectrum of the major β-carotene component, however, shows an obvious '*cis*-peak' around 341 nm, two intense peaks around 444 nm and 471 nm, which are different from that of the all-*trans* standard but consistent with that of the 9-*cis* standard within experimental error. These data agree with the reported absorption spectra of all-*trans*- and 9-*cis*-β-carotene isomers [19,20]. According to the relative intensities of the '*cis*-peaks' and positions of the intense peaks of various isomers [19,20], only a 9-*cis* configuration can be assigned to the absorption spectrum of the major β-carotene component extracted from spinach Cyt b_6f complex.

3.3. Resonance Raman spectra of β-carotene of cytochrome b_6f complex

The Raman spectral pattern, especially in the fingerprint region ν_2 (1100–1300 cm^{-1}), is unique for each of the 14 isomers of β-carotene and is widely used as a key in predicting the configuration of β-carotenes [19–22]. Fig. 4A,C and D show the resonance Raman spectra (800–1700 cm^{-1} region, 488 nm excitation) of the native Cyt b_6f in 30 mM β-OG, the 9-*cis*- and the all-*trans*-β-carotene standards in hexane, respectively. It is shown that native Cyt b_6f (Fig. 4A) exhibits typical Raman bands of β-carotene that are usually grouped into four regions (ν_1 to ν_4) [20]. The Raman spectrum of native Cyt b_6f (Fig. 4A) matches the 9-*cis* standard (Fig. 4C). They have the same location (1530 cm^{-1}) of the ν_1 band and essentially the same spectral pattern (both have one major band at 1156 cm^{-1} and three minor bands at similar positions) in the ν_2 region. They are different from the all-*trans* standard (Fig. 4D) in the position of the ν_1 band and the relative intensity of the Raman band around 1134 cm^{-1}. Compared to the all-*trans* standard, they have an 8 cm^{-1} up-shift of the ν_1 band and an obvious weak Raman band around 1134 cm^{-1}. The up-shift of the ν_1 band, which arises from a C=C stretching mode, is an indication of the presence of '*cis*' configuration in the structure of β-carotene. The 1134 cm^{-1} band is almost undetectable in the all-*trans* isomer, while it increasingly becomes more intense in the *cis* isomers from 7-*cis* to 9-*cis*. Among the other 12 isomers isomerized from the all-*trans*-β-carotene at 190°C [19],

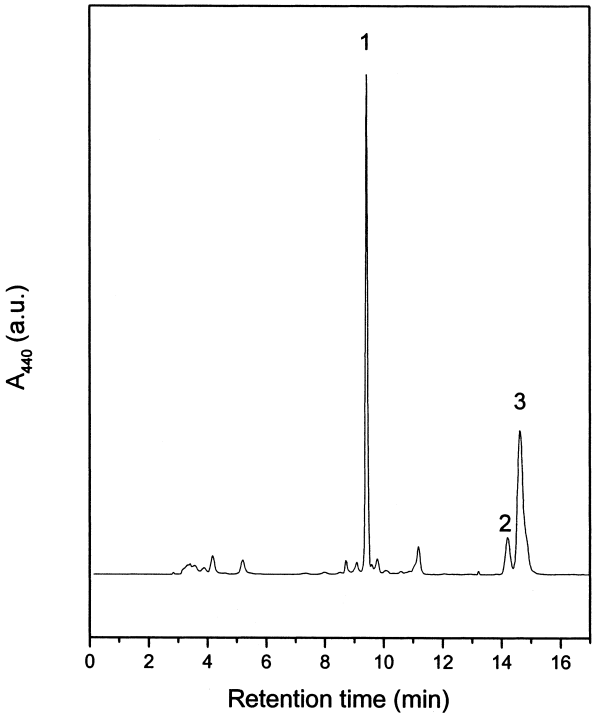

Fig. 2. HPLC chromatogram of the resolved pigments extracted from Cyt b_6f complex preparation. Peak 1, Chl *a*; peak 2, minor β-carotene; peak 3, major β-carotene component.

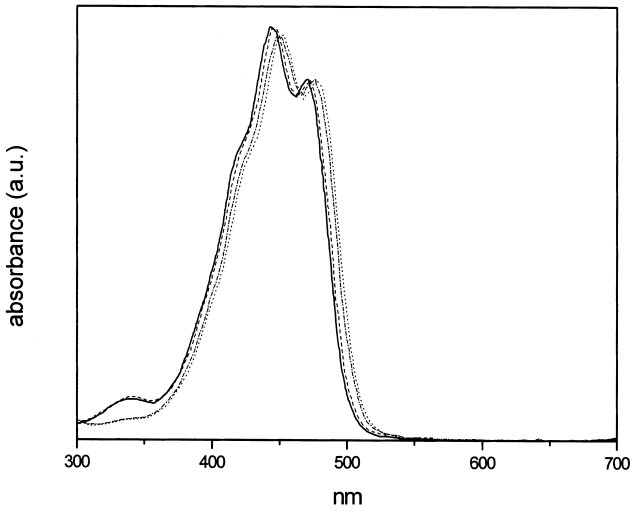

Fig. 3. Room temperature absorption spectra of the major (solid line) and minor (dash-dotted line) β-carotene components resolved by HPLC in Fig. 2, and of 9-*cis*- (dashed line) and all-*trans*- (dotted line) β-carotene standards.

only 7-*cis* and No. 16 (an unidentified isomer in [19]) show a Raman spectral pattern comparable to the β-carotene in Cyt b_6f. However, the 7-*cis* isomer has an additional characteristic weak Raman band at 1274 cm^{-1} and the same low relative intensity of the '*cis*-peak' as that of the all-*trans* isomer in the absorption spectrum. The absorbance maximum at the longest wavelength in the absorption spectrum of No. 16 (maximum at 464 nm [19]) is 7 nm down away from that of β-carotene in Cyt b_6f (maximum at 471 nm, Fig. 3). Therefore, the configuration of β-carotene in native Cyt b_6f complex can be clearly assigned to 9-*cis* from its resonance Raman spectrum. The Raman spectrum of β-carotene of Cyt b_6f in this work is in agreement with that generated by 441.6 nm excitation [15]. However, their assignment of all-*trans* configuration [15], which was based solely on the absolute position of the ν_1 band without comparison with that of the all-*trans* standard at the same experimental condition, is proved to be inappropriate by spectral comparison with the all-*trans* standard in this work.

3.4. The binding of β-carotene in native cytochrome b_6f complex

The Raman band around 1005 cm^{-1} (ν_3 band), which is originated from the methyl in-plane rockings and is not structurally sensitive, had been used as reference to measure the relative intensity of other bands [20–22]. It is noted that the relative intensities of the ν_2 and the ν_1 bands of the 9-*cis* standard in hexane (Fig. 4C) increased more than 35% compared to that in native Cyt b_6f (Fig. 4A). The spectral difference in these two samples should originate from a conformational or environmental difference between the β-carotene in organic solvent and in the native protein. When the native Cyt b_6f is disrupted by SDS, a 14% increase of the relative intensities of these bands is also observed (Fig. 4B). SDS can eliminate the CD signal of β-carotene in this complex (data not shown) and most likely partitions the β-carotene into the micelle of SDS. The relative intensity of the Raman bands around 960 cm^{-1} (ν_4 band, C–H out-of-plane wag) was extensively used to predict the symmetry (planar or twisted) of the conjugated backbone of carotenoids [20–24]. The 9-*cis* standard in hexane (Fig. 4C) exhibits a small

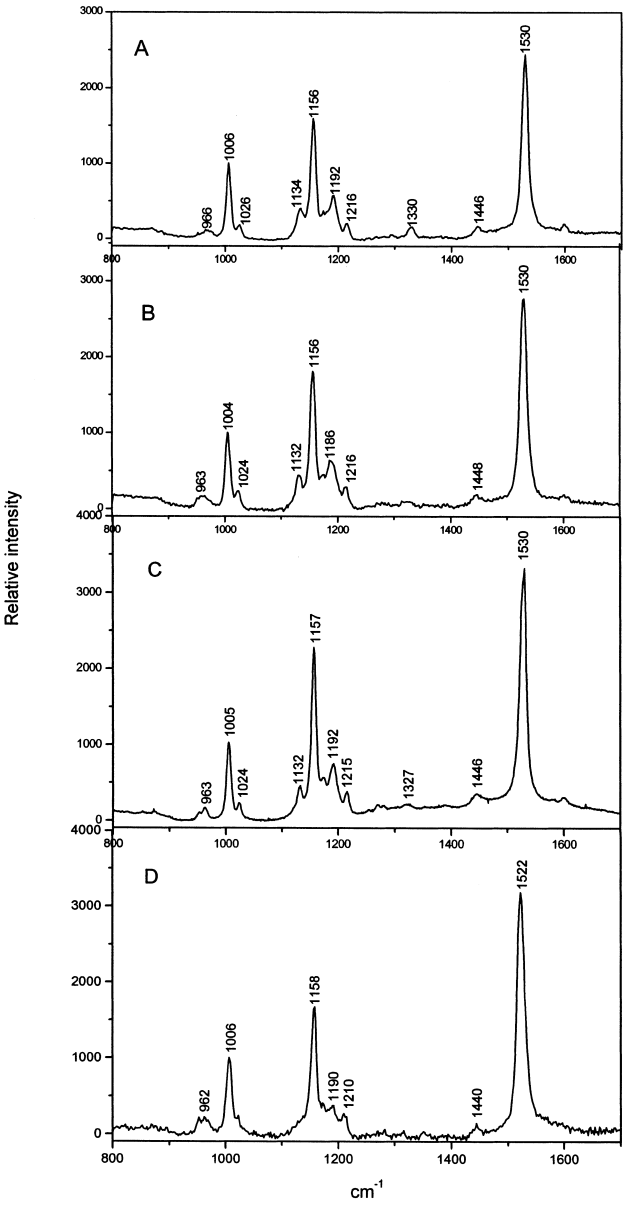

Fig. 4. The resonance Raman spectra (488 nm excitation) of native (A) and SDS-denatured (B) Cyt b_6f, and of 9-*cis*- (C) and all-*trans*- (D) β-carotene standards in hexane.

single band with a shoulder in this region, which is typical for the backbone in planar form. The very weak signal of ν_4 bands in the spectrum of native Cyt b_6f (Fig. 4A) seems to agree with a planar backbone. However, it must be noted that 488 nm may not be a good excitation wavelength to address this question. More extensive Raman spectral comparison between native Cyt b_6f and the 9-*cis* standard or SDS-denatured Cyt b_6f with variation in excita-

tion wavelength and change in medium may reveal more information about the binding state of β-carotene in native Cyt b_6f.

The consistent results obtained from the samples of β-carotene in native Cyt b_6f, in SDS-denatured Cyt b_6f and in organic solvent in this work indicate that the 9-*cis* isomer is a very stable component. This agrees with a previous report [19] that the population of 9-*cis*-β-carotene (22.4%) is only second to the all-*trans* isomer (26.7%) in various isomers isomerized from the all-*trans*-β-carotene at 190°C.

4. Discussion

From the absorption and Raman spectral comparisons of β-carotene in Cyt b_6f with 9-*cis*- and all-*trans*-β-carotene standards in this work and the reported isomers [19,20], it is safely concluded that the β-carotene in Cyt b_6f complex predominantly takes a 9-*cis* configuration in both dissociated and native states. Although the presence of 15-*cis*-β-carotene is well documented in the reaction centers of bacteria, no *cis* configuration of β-carotene was reported for the pigment–protein complexes from chloroplasts except one [25] favoring the presence of a 15-*cis* isomer in photosystem (PS) II reaction centers of spinach. This suggests that the presence of 9-*cis*-β-carotene is unique to the Cyt b_6f complex. It is not likely due to contamination from other pigment–proteins. This further confirms previous reports [7,14] that the carotenoid is an authentic component of Cyt b_6f complex. The presence of a small amount of all-*trans*-β-carotene could be merely contaminant. It is also possible that some 9-*cis*-β-carotene in Cyt b_6f was replaced by free all-*trans*-β-carotene released from other pigment–proteins during the process of detergent extraction and purification of Cyt b_6f. It is noted that the contents of carotenoid in most isolated Cyt b_6f complexes [4,7–10,14] are significantly less than one molecule per Cyt *f*, except that from *Mastigocladus laminosus* [7] which is close to 1.

The reason for the predominance of a 9-*cis*- but not an all-*trans*-β-carotene in the Cyt b_6f complex is unclear at present. 9-*cis*-β-carotene was proved to have higher antioxidant potency than that of all-*trans*-β-carotene in vitro [26]. All-*trans* carotenoids were associated with a good efficiency of light energy transfer in antenna proteins, while 15-*cis* isomers were related with a photoprotective function in reaction centers [27]. A mechanism of triplet transfer from the excited state of the chlorophyll to the carotenoid was proposed for the observed photoprotective function of carotenoids in Cyt b_6f complexes [7]. However, a different mechanism that the quantum yield of Chl triplet formation is significantly lowered by the protein environment without triplet transfer between Chl and carotenoid was observed in Cyt b_6f complex of *Synechocystis* PCC6803 [11]. It was recently reported that Cyt b_6f, specifically the Rieske iron–sulfur center, is one major site of singlet oxygen formation that results in photoinhibition in PS II in spinach thylakoids [28]. The discrepancy between the above two reports [11,7] could be removed if singlet oxygen can actually be produced in Cyt b_6f without the involvement of Chl *a*. It is possible that the major role of 9-*cis*-β-carotene in spinach Cyt b_6f complex may be not to quench the Chl *a* triplet but to act as an effective antioxidant to shield the Chl *a* or other components from the attack by singlet oxygen generated from the Rieske iron–sulfur center or somewhere else.

Acknowledgements

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