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Resonance Raman spectroscopy of carotenoids in Photosystem I particles

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Abstract

Low-temperature resonance Raman (RR) spectroscopy was used for the first time to study the spectral properties, binding sites and composition of major carotenoids in spinach Photosystem I (PSI) particles. Excitation was provided by an argon ion laser at 457.9, 476.5, 488, 496.5, 502 and 514.5 nm. Raman spectra contained the four known groups of bands characteristic for carotenoids (called from ν_1 to ν_4). Upon 514.5, 496.5 and 476.5 nm excitations, the ν_1–ν_3 frequencies coincided with those established for lutein. Spectrum upon 502-nm excitation could be assigned to originate from violaxanthin, at 488 nm to 9-*cis* neoxanthin, and at 457.9 nm to β-carotene and 9-*cis* neoxanthin. The overall configuration and composition of these bound carotenoid molecules in Photosystem I particles were compared with the composition of pigment extracts from the same PSI particles dissolved in pyridine, as well as to configuration in the main chlorophyll *a*/*b* light-harvesting protein complex of photosystem II. The absorption transitions for lutein, violaxanthin and 9-*cis* neoxanthin in spinach photosystem I particles are characterized, and the binding sites of lutein and neoxanthin are discussed. Resonance Raman data suggest that β-carotene molecules are also present in all-*trans* and, probably, in 9-*cis* configurations.

Keywords: Resonance Raman spectroscopy; Carotenoids; Photosystem I particles

1. Introduction

The principal task of photosynthesis is the harvesting of light energy and its transformation into chemical energy. Light harvesting by green plants requires two photosystems located in thylakoid membranes—the main complexes of photosystem I (PSI) and photosystem II (PSII). Each photosystem possesses its own light-harvesting complex, core antenna complex and reaction centers [1]. Almost all photosynthetic pigments—mainly chlorophyll *a* and *b* and carotenoids—are bound to different protein subunits of both photosystems and light-harvesting complexes.

PSI of higher plants is a supramolecular pigment–protein complex, located in nonappressed regions of thylakoid membranes. It consists of chlorophyll (chl) *a* binding core complex (PSI core), where the charge separation takes place, and chlorophyll *a*/*b* binding peripheral light-harvesting complex (LHCI) located on one side of the core [1–3]. The PSI core binds approximately 90–100 chl molecules and 14–20 β-carotene molecules [1–3]. LHCI consists of four polypeptides, which bind chl *a* and all chl *b* and xanthophylls [4]. It has been reported that, in barley thylakoids, LHCI binds about 110 chlorophyll *a*+*b* molecules and approximately 20 xanthophyll molecules [3]. The chlorophyll composition of LHCI has been intensively studied due to the particular interest in the presence of minor, red-absorbing chlorophyll forms [2,3,5].

The knowledge of the carotenoid composition of light-harvesting complexes is important for understanding the detailed mechanisms of their main function to absorb light and funnel the excitation energy to the core complexes. During the past decade, the xanthophyll composition and configuration in light-harvesting complexes of photosystem II (LHCII) has been studied in great detail [6–11]. However, little is known about the spectral properties, conformational state and content of carotenoids in LHCI, probably due to

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difficulties of its purification [12,13]. Data available about the carotenoid composition of the PSI complex are scarce and controversial [12–15]. Lee and Thornber [14] have reported a relatively low content of neoxanthin and substantial amount of lutein, violaxanthin and β-carotene in PSI holocomplex and of LHCI and PSI core complexes isolated from barley (*Hordeum vulgare*) leaves. A relatively low amount of neoxanthin and higher levels of lutein, violaxanthin and some β-carotene have been obtained for PSI fraction from *Arabidopsis thaliana* and pea leaves [13,15]. Neoxanthin has not been detected in the tomato LHCI [12], and as far as we know, there are no data available concerning the carotenoid composition of spinach PSI.

In addition to their main antenna function, carotenoid molecules serve as effective quenchers of chlorophyll triplet states, thus avoiding the generation of harmful reactive oxygen species [16]. An important function of xanthophylls is the structural stabilization of the complexes in which they are incorporated and especially in the three-dimensional structure of light-harvesting complexes of higher plants [17,18]. Effective interaction with chlorophyll raises the question about the precise positions of carotenoid molecules in the pigment–protein bed of main pigment–protein complexes and the electronic levels of their ground and excited states [19].

Resonance Raman (RR) spectroscopy has been proven to be a nondestructive method providing precise information on the type and conformation of carotenoid molecules in multipigment systems [6–11], since carotenoid spectra may be selectively obtained when the excitation wavelengths are longer than 450 nm [19]. Specific absorption maximum positions of lutein, neoxanthin, violaxanthin and zeaxanthin have been identified in LHCII by RR spectroscopy, combined with low-temperature absorption spectroscopy [7,10,11], despite the fact that, in the 400–500 nm region, the absorption of different carotenoids is overlapped. The incorporation of carotenoid molecules in the chlorophyll–protein environment in antenna complexes changes mainly their electronic properties and configuration. Having in mind that the helix protein structure of LHCI and LHCII is quite similar [18,20,21], it is reasonable to assume that electronic transitions of carotenoids in LHCI are nearly the same as in LHCII. When the excitation by narrow laser lines coincides with carotenoid absorption band, a 10^6-fold increase in its Raman signal is achieved relative to the scattering due to any other molecules present [19], and the spectrum of this carotenoid is observed almost exclusively. Thus, the RR technique provides possibility to extract the signal from a specific bound carotenoid in complex systems. For thylakoid membranes, recently, we have showed that, upon laser excitation in the 460–500 nm region, the major xanthophylls belonging to LHCII were excited [22].

In the present investigation Photosystem I particles isolated from spinach leaves by mild digitonin treatment were studied by means of 77K fluorescence and resonance Raman spectroscopy. The aim was to reveal the spectral properties and composition of carotenoids in spinach PSI particles. The spectra obtained were analyzed, and the bands were assigned to different carotenoid molecules dominating the corresponding spectrum. Analysis was made on the basis of the selectivity of resonance Raman signal and the strong dependence of the position of the most intensive band in carotenoid RR spectra on the excitation lines established by Ruban et al. in Ref. [7]. Data obtained for PSI particles were compared with the spectra of pigment extracts from the same PSI particles dissolved in pyridine and with LHCII [6–11,22]. The absorption transitions for lutein, violaxanthin and 9-*cis* neoxanthin in spinach Photosystem I particles are characterized, and the binding sites of lutein and neoxanthin are discussed. Resonance Raman data suggest that β-carotene molecules are also present in all-*trans* and, probably, in 9-*cis* configurations.

2. Experimental

Photosystem I particles were isolated from spinach chloroplasts by mild digitonin treatment [23]. The submembrane particles obtained by this method originated mainly from stroma-exposed regions of thylakoids and consist of PSI-core complex and LHCI. Contaminations with PSII and LHCII are very low, as proved by the chlorophyll *a*/*b* ratio and 77K fluorescence emission spectra. Chl *a*/*b* ratio varied in different experiments but, usually, was more than 5–6. Isolated PSI particles were resuspended in buffer containing 10 mM Tricine–KOH (pH 7.8), 20 mM NaCl, 20 mM KCl and 5 mM $MgCl_2$.

Pigment extraction of spinach PSI particles was performed as described by Ikegami et al. [24], with diethyl ether. Prior to ether extraction, PSI particles were repeatedly washed with distilled water to remove salts and detergent. The ether-treated material was dried under N_2 flux and then dissolved to appropriate concentration in pyridine. The latter was chosen, as its polarizability was close to that of lipid and membrane protein environments [7]. LHCII was isolated as described earlier [25,26].

The chlorophyll content of the samples was adjusted to 10 and 200 μg ml^{-1} for fluorescence and Raman measurements, respectively. The chlorophyll concentration was measured in 80% acetone according to Lichtenthaler [27].

RR and fluorescence spectra at 77K were obtained in a translucent Dewar using a double monochromator spectrometer (model 1403; Spex Industries, Edison, NJ, USA), equipped with a cooled photomultiplier tube (model R943, Hamamatsu Photonics, K. K., Shizuoka, Japan). Excitation was provided by an argon ion laser (ILA 190) at 514.5, 502, 496.5, 488, 476.5 and 457.9 nm. Principal argon-laser plasma lines were used for frequency calibration. At least three independent experiments were performed. Five to 10 successive Raman spectra were averaged for each experiment.

3. Results and discussion

The steady-state emission spectra of PSI particles at 77K upon different excitations are shown in Fig. 1. The maximum of all emission spectra is located nearly 734 nm. They are typical for plant PSI fluorescence at low temperature, dominated by the emission from LHCI-730, with emission maximum at 730–735 nm [2]. The very low intensity shoulder around 680 nm could be assigned to LHCI-680 [2,5,12].

Laser lines at 514.5, 502, 496.5, 488, 476.5 and 457.9 nm were used to excite selectively carotenoid molecules in multipigment PSI particles. The spectra shown in Fig. 2 contained four known groups of bands (called from ν_1 to ν_4) characteristic for carotenoids [19,28]. The bands have been assigned [19,28] as follows: ν_1 to C=C bonds in phase stretching vibrations; ν_2 to C_{14}–C_{15} stretches coupled to C_{15}–H in plane bending; ν_3 to methyl CH_3 in plane rocking vibrations; and ν_4 to C–H out of plane bending modes coupled with C_7=C_8 torsion.

The most intensive band in the carotenoid resonance Raman spectrum (called ν_1) is located at ~1530 cm^{-1}. Its precise position is strongly sensitive to both the number of conjugated double bonds (the length of polyene chain) and the carotenoid configuration [7,11,19,28]. Each additional double bond produces a ~3 cm^{-1} downshift of this parameter [19,28] and, therefore, can be used to identify the specific carotenoid absorption transitions in complexes containing carotenoids with a distinguished number of conjugated double bonds. Ruban et al. [7] have found that the ν_1 position depends strongly on the excitation lines, being quite different for each carotenoid (Fig. 3). It has been shown [7,10] that in LHCII scanning of Raman excitation through the 450–500 nm region induces changes of the ν_1 band from the frequency expected for 9-*cis* neoxanthin to that expected for lutein, indicating that the contribution of these carotenoids alternatively dominates the RR spectra. Thus, by ν_1 position, it is possible to recognize which carotenoid dominates the RR spectrum obtained at a given wavelength, matching the absorption of this carotenoid molecule.

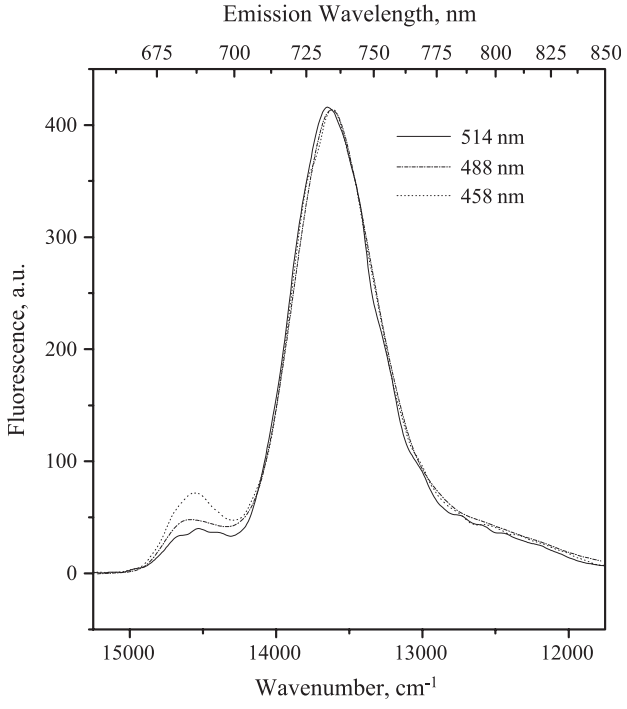

Fig. 1. 77K fluorescence emission spectra of PSI particles at different excitation wavelengths. Chlorophyll concentration was 10 μg Chl/ml.

In PSI particles, excitation lines at 514.5, 502, 496.5, 488, 476.5 and 457.9 nm yielded the following ν_1 positions: 1526.5, 1531, 1528.5, 1533, 1527 and 1533 cm^{-1}, respectively (Fig. 4A). They are presented as circles in Fig. 3 and were very similar to those of lutein at 514.5 and 476.5 nm, of violaxanthin at 502 nm, and of neoxanthin at 488 and 457.9 nm. In addition, the ν_1 bandwidths in PSI particles at all excitation lines, except at 457.9 nm, were between 12.5 and 14.6 cm^{-1}, equal or slightly smaller than those of corresponding carotenoids in pyridine [7], confirming that, at these wavelengths, only one type of carotenoid contributes to the band. The lack of expected intensive ν_1 band at 1522–1524 cm^{-1}, a position characteristic for all-*trans* β-carotene [7,19,28,29], was surprising, taking into account its abundance in the core complex of PSI [1–3]. However, the discernible shoulder at smaller wave numbers and wider bandwidth (15.4 cm^{-1}) at 457.9 nm excitation is indicative for the presence of some all-*trans* β-carotene. Its very small contribution could be explained by the assumption that, at these wavelengths in the PSI particles, the excitations did not reach the core pigments and only the major xanthophylls belonging to LHCI were excited. Most probably, the formed LHCI belt [20] surrounding the PSI core could shield the core β-carotene molecules, thus hindering their direct excitation. To test this possibility, we compared the spectra of PSI particles with the spectra of pigment extracts from the same PSI particles, dissolved in pyridine (Fig. 4B). In the latter, the contribution of all-*trans* β-carotene is more clearly pronounced at 457.9-nm excitation (curves 1 and 2 in Fig. 4B) compared with 488 nm (curves 3 and 4 in Fig. 4B), but still not so great as we expected. Results obtained confirmed that the LHCI belt shielded some all-*trans* β-carotene molecules. However, this could be not the only reason for the ν_1 band small intensity at 1522–1524 cm^{-1}. There are several reasons that could explain these experimental results. It is quite possible that the RR spectra are dominated by xanthophylls because, at the wavelengths used, the β-carotene is out of resonance. The other explanation could be the known upshift of the ν_1 band [19] for β-carotene induced by *cis* isomerization, which is 1 and 5 cm^{-1} for the 7- and 9-*cis*-isomers and becomes up to 13 cm^{-1} for central isomers [28,29]. It can be speculated, therefore, that not all of β-carotene molecules in PSI are in all-trans configuration state, thus shifting ν_1 band at higher wavenumbers. The presence of 15-*cis* β-carotene was found in the reaction center of spinach PSI [30] by

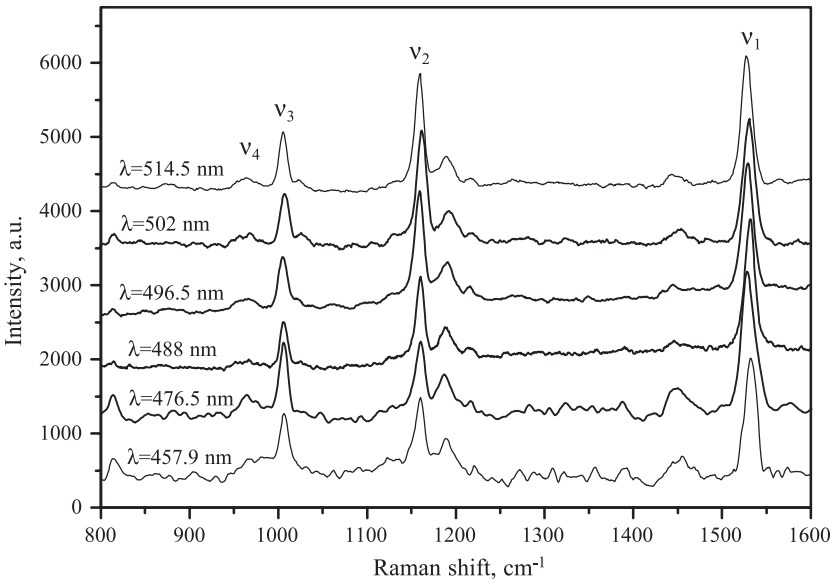

Fig. 2. Resonance Raman spectra upon 514.5, 502, 496.5, 488, 476.5 and 457.9 nm excitation of spinach Photosystem I particles. Chlorophyll concentration was 200 μg Chl/ml.

high-pressure liquid chromatography. However, our experimental data (Fig. 2) showed relatively weak intensity around 1134 cm^{-1} in the *cis*-isomerization fingerprint region ν_2 (1100–1300 cm^{-1}) [19,28,29]. It leads us to the conclusion that the presence of 15-*cis*-isomers in PSI particles is not likely. The contribution of a very stable component 9-*cis* β-carotene [31,32] is not excluded, since the location of its ν_1 band, 1530 cm^{-1}, is near to the observed position and its Raman band around 1134 cm^{-1} is relatively weak. Having in mind the complicated nature of

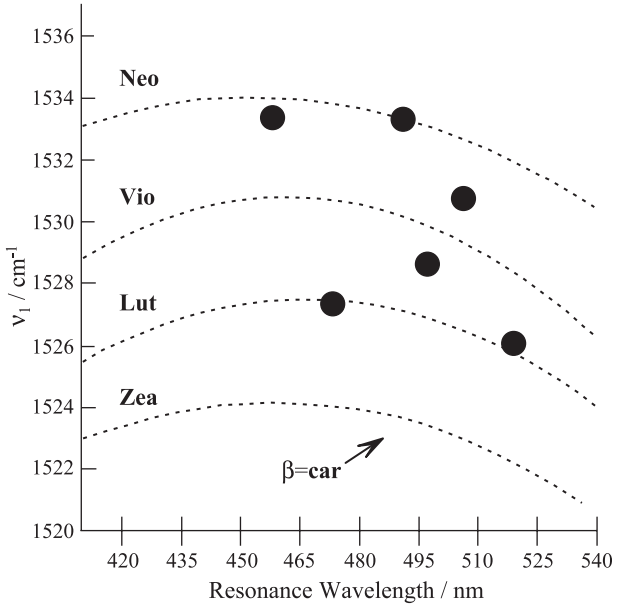

Fig. 3. Dependence of ν_1 maximum position on excitation wavelength for the main xanthophylls and β-carotene (dashed lines taken with author's permission from Ruban et al. [7]). Circles represent the experimental data for PSI particles.

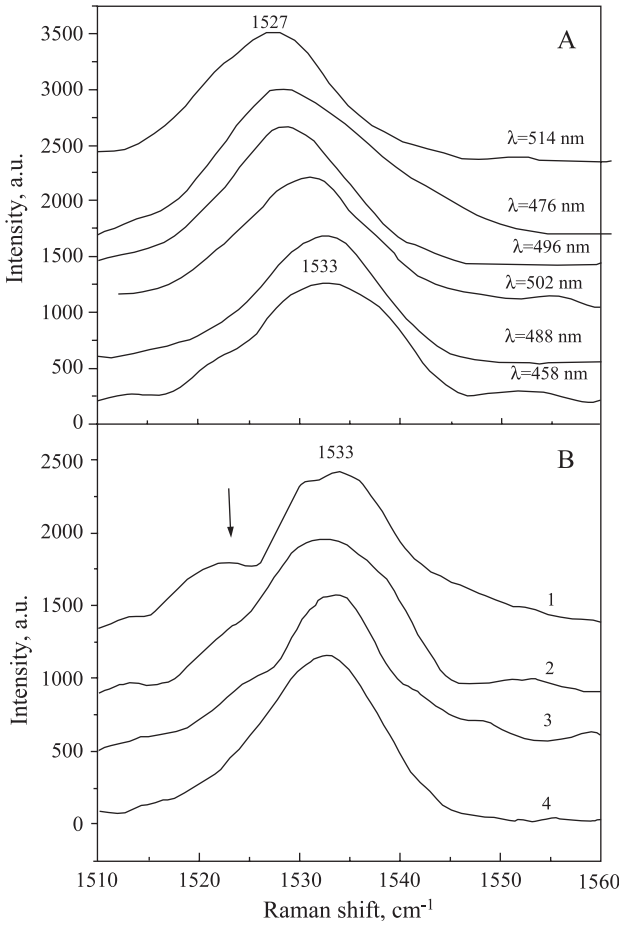

Fig. 4. (A) ν_1 Region of the resonance Raman spectra of spinach PSI particles upon the excitation wavelength used. (B) Spectra of the pigment extracts from the same PSI particles, dissolved in pyridine (traces 1 and 3) compared with the ones of spinach PSI particles (traces 2 and 4) upon 457.9 and 488 nm excitation, correspondingly.

our PSI preparations, it is reasonable to compare their RR spectra with those of core PSII β-carotene molecules. Very recently, only one paper concerning the resonance Raman spectroscopy of carotenoids in PSII core complexes has appeared [33]. The results are consistent with a predominance of the all-trans configuration for the carotenoids in the PSII core complex. In higher plant's PSII reaction center, where two β-carotene molecules with different electronic properties are presented [34], the RR spectra are also in consistence with the conclusion of an all-trans configuration for both carotenes in neutral state [34,35].

At 496.5-nm excitation, the ν_1 position, 1528.5 cm^{-1}, was between the positions of lutein and violaxanthin. Its narrow bandwidth confirmed that only one xanthophyll was excited. To select the type, we compared the features in the ν_3 region. The corresponding ν_3 position difference between them (about 4 cm^{-1} [7]) provided evidence that, namely, the lutein was excited.

Our results suggest that the absorption transitions for lutein should be located at 514.5, 496.5 and 476.5 nm for violaxanthin at 502 nm and for neoxanthin at 488 and 457.9 nm, respectively. In LHCII, it has been found that 0–0, 0–1 and 0–2 transitions of neoxanthin were located around 486, 457 and 430 nm, respectively [7,10]. Therefore, the 0–0, 0–1 transitions coincide with the transitions that we found in PSI particles. The lutein absorption in LHCII depends on the oligomerization state of the complex [7,10]. In monomeric LHCII, the absorption bands were found at 495, 466 and 437 nm, whilst in trimeric LHCII, an additional broad band at 510 nm was found [7,10]. The lutein absorption transitions observed in our study are in good agreement with those reported for lutein in trimeric LHCII [7,10]. Absorption at 502 nm falls in the absorption region of both violaxanthin and zeaxanthin. Violaxanthin in LHCII exhibits two populations, one with absorbing transition (0–0) at 497 nm and another one absorbing at 488 nm [7]. The zeaxanthin absorption has been found to be from 503 to 532 nm [7,11]. However, both ν_1 and ν_3 positions at 1524 and 1003 cm^{-1}, respectively, are too far from the positions we observed at 1531 and 1007 cm^{-1}. Moreover, for the preparation of PSI particles, we used dark adapted leaves, and it has been shown that, in such preparation, no zeaxanthin is present [14]. Therefore, it is reasonable to assign the absorption at 502 nm to violaxanthin.

To analyze the binding sites of lutein and neoxanthin in PSI, we compared their RR spectra excited at 514.5 and 488 nm, respectively, with the spectra of aggregated LHCII and LHCII trimers. As could be expected, differences in the spectra between the complexes were found only in the region of ν_4, since the binding of carotenoids to the proteins in antenna complexes affects mainly the ν_4 band without changing the ν_1 band position [7,10,11]. Fig. 5 displays the ν_4 band of PSI particles (trace 1 in Fig. 5), aggregated LHCII (trace 2 in Fig. 5A) and trimeric LHCII (trace 3 in Fig. 5A and trace 2 in Fig. 5B). Transitions in the ν_4 region become allowed only when carotenoid molecules are

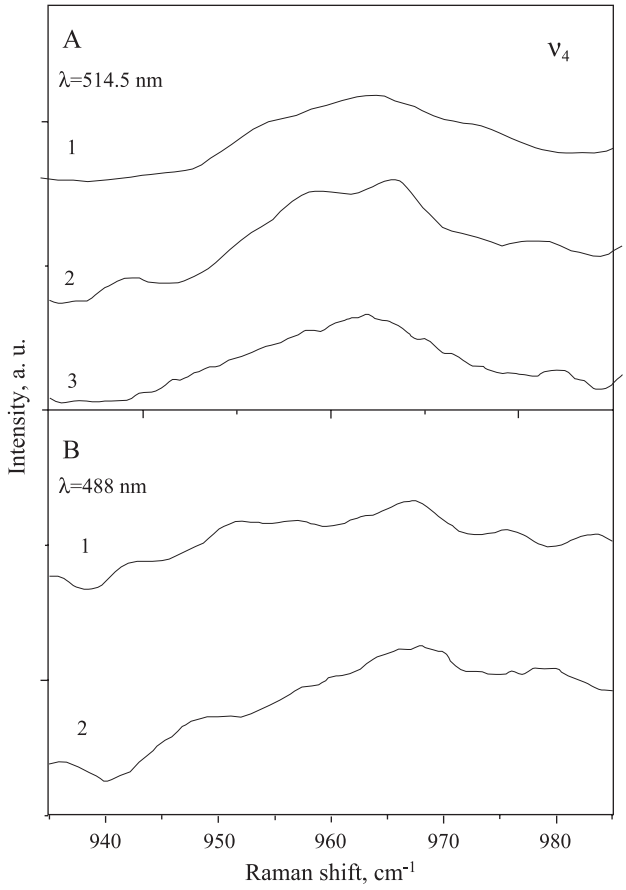

Fig. 5. Resonance Raman spectra upon 514.5 (A) and 488 nm (B) excitation of spinach PSI particles (trace 1) in the ν_4 region. Spectra of isolated aggregated LCH II (trace 2 in A) and LHCII trimers (trace 3 in Panel A and trace 2 in Panel B) are also shown.

distorted from their planar configuration [7,11,19,36]. It has been established [7,10,11] that the binding of carotenoids to proteins in antenna complexes leads to specific changes in this region. The similar structure of the lutein ν_4 band in PSI particles and LHCII trimers supposes that lutein, absorbing at 514.5 nm, is included in the same manner in LHCI as in trimeric LHCII (compare traces 1 and 3 in Fig. 5A). Thus, it is reasonable to assume that lutein should occupy the so-named L1 site preserved for lutein in chl *a*/chl *b* binding proteins of both PS [20,21,37,38]. The quite similar helix protein structure of LHCI and LHCII [18,20,21] allows us to suppose that the lutein molecules in PSI interact with the protein helices through van der Waals contacts and hydrogen bonds in the similar manner as it was shown for LHCII two luteins [21]. Most likely, these interactions lead to the observed distortion of the lutein's polyenic chains and stabilize the three-dimensional structure of PSI complexes. The small differences in the structure of the ν_4 band of neoxanthin found in both antenna complexes (compare traces 1 and 2 in Fig. 5B) are indicative that neoxanthin environment differs slightly in LHCI compared with LHCII. Probably, in LHCI, neoxanthin does not occupy N1 site, as in LHCII [9], but L2 or V1 sites.

The analysis of RR data for PSI particles obtained in this work, and their comparison with the spectra of pigment extracts from the same PSI particles, aggregated LHCII and LHCII trimers, allow us to characterize the spectral properties, binding sites and composition of major carotenoids in spinach Photosystem I particles. The absorption transitions for lutein, violaxanthin and 9-*cis* neoxanthin in spinach Photosystem I particles are characterized, and the binding sites of lutein and neoxanthin are discussed. It was suggested that β-carotene molecules are also present in all-*trans* and, probably, in 9-*cis* configurations. Although previous reports have indicated very low [13–15] or no detectable [12,39] amount of neoxanthin in LHCI, the high selectivity and sensitivity of resonance Raman spectroscopy allowed us to detect it. The unexpected small contribution of β-carotene could be explained by the assumption that the RR spectra, at the wavelengths used, are dominated by xanthophylls because β-carotene is out of resonance and of the presence of some 9-*cis* β-carotene isomers, and/or that, in PSI particles, the LHCI belt shields the core β-carotene molecules.

Divergent results from different laboratories about carotenoid content in PSI illustrate the difficulty of correct estimation of pigment stoichiometry and pigment content of pigment–protein complexes. This is mainly due to the probability of artifactual loss of pigment during membrane solubilization and complex isolation. It has been shown for LHCII that pigment content varies with isolation procedure [40]. That is why the detection of higher neoxanthin levels and lower β-carotene content in our spinach PSI particles estimated by their RR spectra is not surprising. Moreover, the differences may be due to different plant species and/or differences in growth light regimes.

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