Fluorescence of native and carotenoid-depleted LH2 from *Chromatium minutissimum*, originating from simultaneous two-photon absorption in the spectral range of the presumed (optically 'dark') S_1 state of carotenoids

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Abstract Native and carotenoid-depleted peripheral purple bacterial light-harvesting complex (LH2) were investigated by simultaneous two-photon excited (between 1300–1500 nm) fluorescence (TPF). TPF results from direct bacteriochlorophyll excitation in both samples. The spectral position of the $2A_g^-$ state of rhodopsin is indicated by a *diminuition* of the bacteriochlorophyll TPF in native LH2. In conclusion, comparison to carotenoid-depleted samples is a *conditio sine qua non* for unambiguous interpretation of similar experiments.

Key words: Carotenoid-less; Excitation energy transfer; Rhodopsin; Two-photon excited fluorescence; *Chromatium minutissimum*

1. Introduction

Carotenoids play three essential roles in (bacterial) photosynthesis: (i) as accessory light-harvesting pigments in the 400–550 nm region where (bacterio)chlorophyll, (B)Chl, absorption is low, (ii) as protective agents against potentially harmful (B)Chl triplet states and reactive oxygen species, and (iii) as integral structural components of pigment–protein complexes. Due to advances in spectroscopy, especially the first two issues have become accessible over the past two decades, as reviewed recently [1–3].

Strong absorption of carotenoids in the visible region is the result of an electronic transition from the ground state, S_0 ($1^1A_g^-$), to the higher singlet state S_3 ($1^1B_u^+$). Note, that due to recent discoveries, e.g. [4], the nomenclature for carotenoid excited states has to be revised. Therefore, the allowed transition – previously referred to as $S_0 \rightarrow S_2$ – is designated $S_0 \rightarrow S_3$. The first excited singlet state, S_1 ($2^1A_g^-$), and the only recently experimentally detected second excited singlet state, S_2 ($1^1B_u^-$), of carotenoids [4] are optically 'dark', i.e. their radiative combination with S_0 is forbidden by the parity ($g \leftarrow // \rightarrow g$) and pseudoparity ($- \leftarrow // \rightarrow -$) selection rules [5–7]. Descriptions by these terms are strictly valid only for C_{2h} symmetric molecules, e.g. for all-*trans* polyenes. However, highly (and even unsymmetrically) substituted carotenoids with extended chains of conjugated double bonds, which are common in photosynthetic pigment–protein complexes, are apparently described well, too.

Following excitation to the carotenoid S_3 state, rapid internal conversion (IC) to S_1 occurs (within 100–200 fs) in solution as well as in pigment–protein complexes [8,9]. Thus, to account for efficient excitation energy transfer (EET) from light-harvesting carotenoids to (B)Chl, a significant portion of the excitation energy is assumed to flow through the carotenoid S_1 state [10]; the respective role of S_2 is not investigated in comparable detail, yet.

To elucidate the role of all three (via S_1, S_2, S_3) carotenoid-to-(B)Chl EET channels, their selective excitation would be highly desirable, which, however, requires knowledge of the energetic locations of the 'dark' S_1 and S_2 states. Considerable effort has been devoted to determine the S_1 energies of relevant carotenoids (for a recent review see [1]). A novel approach is provided by XANES and soft X-ray optical double-resonance spectroscopy [11,12]. However, results obtained with carotenoids in photosynthetic pigment–protein complexes are still very scarce.

In principle, one-photon forbidden $1^1A_g \rightarrow 2^1A_g$ transitions are the exemplary targets of simultaneous two-photon absorption (TPA) spectroscopy, as shown theoretically [5] and experimentally [13] already 15 years ago for polyenes including carotenoids. However, this technique has well-known inherent problems [1,13], complicating a straightforward determination of the S_1 energy of carotenoids. A modification of the technique, adapted to the carotenoid-to-(B)Chl EET issue, has

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Abbreviations: (B)Chl, (bacterio)chlorophyll; EET, excitation energy transfer; IC, internal conversion; LH2, peripheral purple bacterial light-harvesting complex; NIR, near infra-red; TPA, (simultaneous) two-photon absorption; TPF, (simultaneous) two-photon excited fluorescence

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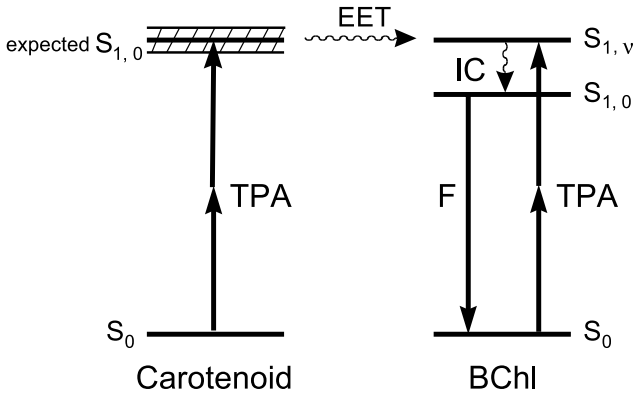

Fig. 1. Diagram representing energy levels and radiative (TPA, F) as well as non-radiative (EET, IC) processes of carotenoids and BChls necessary to describe TPF of LH2. The measuring range (region where carotenoid S_1 is to be expected) is shaded (not to scale). F, fluorescence.

been introduced by Shreve et al. [14,15]. The Chl *a* fluorescence excitation spectrum of thylakoid membranes isolated from *Phaeodactylum tricornutum* was measured in the presumed spectral range of the carotenoid S_1 state using simultaneous TPA of corresponding near infra-red (NIR) pulses (cf. Fig. 1).

A similar approach has been utilized recently to investigate carotenoid excited state processes in bacterial [10] as well as in higher plant [16] antenna complexes. The two-photon excitation spectra of (B)Chl fluorescence, e.g. in the 1100–1500 nm range for peripheral purple bacterial light-harvesting complex (LH2) of *Rhodobacter sphaeroides* [10] and in the 1100–1400 nm range for the higher plant antenna complex, LHC II, [16] revealed broad features near 650 and 600 nm, respectively, that were assigned to the pertinent carotenoid S_1 states. The occurrence of these features was taken as direct evidence for the participation of carotenoid S_1 states in EET processes [10,16].

However, the alternative/additional possibility of direct TPA of (B)Chl has to be considered. In the following, we describe a straightforward approach to differentiate between fluorescence originating from indirect (carotenoid) and direct BChl *a* TPA by comparing the excitation spectra of two-photon BChl *a* fluorescence (TPF) in native LH2 of *Chromatium minutissimum* to those of a corresponding carotenoid-depleted complex. Additionally, TPF excitation spectra were measured for dissolved BChl *a* in the same spectral region. The results clearly demonstrate direct two-photon excitation of BChl *a* in both LH2 samples as well as in solution in this particular region. Moreover, the similarity of the TPF excitation spectra for both complexes demonstrates that BChl *a* dominates TPF excitation even in native LH2.

2. Materials and methods

Cells of *C. minutissimum* (strain MSU) were grown for 3–4 days at 30°C in a luminostate. To obtain carotenoid-less cells, diphenyl amine (0.7 μM) was added to the growth medium [17]. Cells were collected by centrifugation at 5000×*g*. Chromatophores were prepared according to [18]. LH2 was purified by preparative polyacrylamide gel electrophoresis using a home-made apparatus [19]. The carotenoid composition of native LH2 is 80.0% rhodopsin, 9.5% spirilloxanthin, 5.2% anhydrorhodovibrin, 2.9% lycopene and 2.4% dimethylspirilloxanthin [17]. BChl *a* was purified by high-performance liquid chromatography (HPLC) [20]. Absorption spectra were recorded using a Lambda-19 spectrometer (Perkin-Elmer). A Fluorolog FL-112 fluorimeter (Spex) was used to measure steady-state fluorescence spectra. TPF measurements were carried out using a home-made fs spectrometer. The excitation pulses (with center wavelengths between 1300 and 1490 nm) were generated by an optical parametric amplifier, pumped at 790 nm by a Ti:sapphire laser system [21,22]. Pulses of 7–9 μJ (corresponding to $5–7\times10^{13}$ photons per pulse) were used to excite the samples at the desired wavelengths. The pulse duration was measured by auto-correlation yielding approximately 80 fs (full width at half of maximum), which remained fairly constant over the entire tuning range. A rotating cell (optical path length 0.3 mm) contained the respective samples. At the laser repetition rate of 1 kHz, the irradiated sample volume is exchanged after each pulse. Fluorescence was passed through a monochromator and detected by a photomultiplier with GaAs photocathode (R636-10, Hamamatsu). At each excitation wavelength the quadratic dependence of TPF on the excitation intensity was verified. TPF was corrected for excitation pulse energy and pulse duration variations.

3. Results and discussion

Absorption spectra of native and carotenoid-depleted LH2 are shown in Fig. 2. Conventional (one-photon excited) as well as TPF emission spectra of both complexes were virtually identical (data not shown).

We have obtained TPF excitation spectra of BChl *a* from native and carotenoid-depleted LH2 using NIR laser pulses between 1300 and 1490 nm (Fig. 3). In the corresponding one-photon range (650–745 nm) the rhodopsin S_1 state can be expected (vide infra, cf. also Fig. 1). Within the error limits, the BChl *a* TPF intensity is the same for both complexes over almost the entire measuring range. We therefore conclude that the origin of TPF has to be attributed to TPA of BChl *a* itself, and this conclusion is corroborated by the results obtained with BChl *a* in acetone solution, also shown in Fig. 3. (Note that the Q_y absorption of dissolved BChl *a* is blue-shifted in comparison to that of the LH2-bound BChl *a*, as shown also in Fig. 3.) Apparently, the influence of TPA of rhodopsin on BChl *a* TPF is negligibly small in almost the entire measuring range. The only exception is found in the region around 745 nm, which is at the end of the currently accessible measuring range. Here, the TPF intensity of native LH2 is even significantly diminished as compared to that of the carotenoid-depleted complex under identical excitation conditions. This underlines that there is no EET from the carotenoid to BChl in the spectral region under investigation,

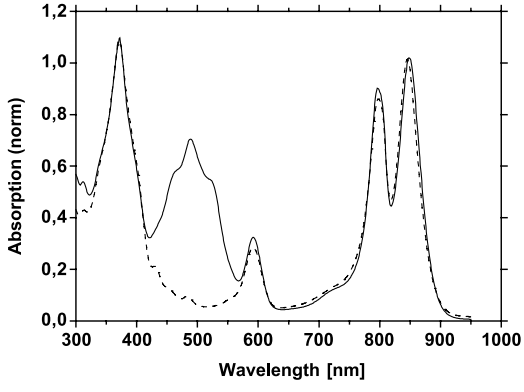

Fig. 2. Absorption spectra of LH2 complexes isolated from native (solid line) and carotenoid-depleted cells (dashed line) of *C. minutissimum*. The spectra are normalized to unity at the long wavelength maximum (850 nm).

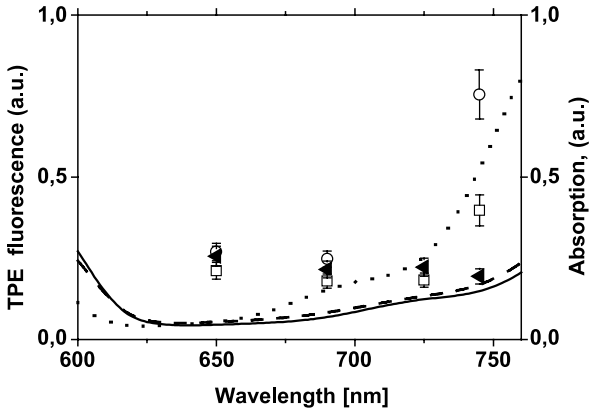

Fig. 3. TPF excitation spectra of LH2 isolated from native (closed triangles) and carotenoid-depleted (open squares) cells of *C. minutissimum*. Additionally, TPF excitation of BChl *a* in acetone is shown (open circles). Fluorescence was detected at 870 nm for LH2 and at 780 nm for dissolved BChl *a*. Each data point represents the mean of 10^3 repetitions. The scale for both LH2 samples is the same, the BChl *a* TPF intensity was normalized to that of the LH2s at 650 nm. For comparison the absorption spectra of LH2 (solid curve – native, dashed curve – carotenoid-depleted) and BChl *a* in acetone (dotted) are presented.

but, on the contrary, that around 745 nm the carotenoid acts as non-linear absorber diminishing the photon density available for TPA of BChl. The phenomenon can be simulated in the framework of a specifically adapted coupled system of photon transport and rate equations for the carotenoid as well as BChl without EET between them. Preliminary results indicate that the non-linear response of the carotenoid at 745 nm results from both, a TPA cross-section larger than that of BChl and an efficient one-photon absorption starting from the TPA excited state. The latter process is well-founded: (i) it terminates in the 20 000 cm^{-1} region, where the $1^1B_u^+$ state of rhodopsin is located ($S_0 \rightarrow S_3$ transition at 500 nm, cf. Fig. 2); (ii) corresponding excited state absorptions have been observed for other carotenoids [23].

For two carotenoids containing 11 conjugated double bonds, viz. lycopene [24] and 5′,6′-dihydro-7′,8′-didehydrospheroidene [25], the S_1 state energies in *n*-hexane solution were determined to be 757.5 ± 5 nm and 755 ± 3 nm, respectively. Even taking possible protein-induced shifts (as observed for the $S_0 \rightarrow S_3$ transition of rhodopsin) into account the carotenoid TPA around 745 nm in LH2 of native *C. minutissimum* can be assigned to S_1 of rhodopsin.

The present results advise to be extremely cautious when interpreting results from two-photon excitation measurements of (B)Chl fluorescence with native (carotenoid-containing) pigment–protein complexes which are not substantiated by comparison to carotenoid-depleted control samples (accessible by mutagenesis, inhibitor treatment or other means). On the other hand, such a comparison allows not only to distinguish between TPA of carotenoids and of (B)Chls, but also to locate the energies of the optically dark carotenoid excited states.

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