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Two-Photon Excitation Spectrum of Fluorescence of the Light-Harvesting Complex B800-850 from *Allochromatium minutissimum* within 1200–1500 (600–750) nm Spectral Range Is Not Carotenoid Mediated[1]

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Abstract—Two types of peripheral light-harvesting complexes LH2 (B800–850) from photosynthetic purple bacterium *Allochromatium minutissimum* were studied. First type containing carotenoids was prepared from wild type cells. The other one was obtained from carotenoid depleted cells grown with diphenylamine. We have shown that under laser femtosecond excitation within absorption 1200–1500 nm wavelength range the two-photon excitation of LH2 complexes takes place. This can be observed as fluorescence of bacteriochlorophyll (BChl) spectral form B850 (BChl molecules of circular aggregate with strong exciton interaction in 850 nm spectral domain). LH2 fluorescence excitation spectra under two-photon excitation are the same for carotenoid-containing and carotenoidless preparations. In both cases the broad band with peak near 1350 (675) nm (FWHM ~ 240 (120) nm) was found. It is concluded that the broad band with peak near 1350 (675) nm in two-photon excitation spectra of LH2 complexes from *Allochromatium minutissimum* cannot be interpreted as two-photon excitation band of the optically forbidden $S_0 \longrightarrow S_1$ transition of carotenoids (rhodopin). Possible nature of this band is discussed.

Key words: *Allochromatium minutissimum*, LH2, two-photon excitation, purple bacteria, carotenoids, bacteriochlorophyll.

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Primary (physical) processes of photosynthesis take place in two main structural components of photosynthetic apparatus of purple bacteria: in a photoactive reaction center (RC) and in a light-harvesting antenna. Pigment-protein complexes localized within inner membranes as two-dimensional lattice are structural basis of both antenna and RC. Light-harvesting "core" complex (LH1 or B875) with absorption band in 875–900 nm region is an inherent part of purple bacteria apparatus. Peripheral light-harvesting complex (LH2 or B800–850) with two absorption bands near 800 and 850 nm may be found in most, but not all purple bacteria. Now X-ray data are available for LH2 complexes [1, 2], for ensemble LH1+RC [3] as well as electron microscopic data for their mutual arrangement in membrane [4]. Bacteriochlorophyll molecules are main pigments of complexes (RC contains also bacteriopheophytin molecules). Auxiliary pigments are presented by carotenoid molecules. These pigments have no covalent links with protein part of complexes and may be removed by organic solvents or detergents.

According to current concepts ([5, 6] and references therein), carotenoids have several functions. Carotenoids increase light harvesting absorbing light quanta and transferring excitation energy to BChl molecules. Carotenoids protect photosynthetic apparatus from photodamage (by quenching of highly reactive triplet states of BChl molecules). Also carotenoids increase structural stability of pigment-protein complexes. Effi-

[1] The article is translated by the authors.
Abbreviations: *Alc. minutissimum*, *Allochromatium minutissimum*; LH2 complex or complex B800-850, peripheral light-harvesting complex with absorption bands near 800 and 850 nm; LH1 complex, core light-harvesting complex with absorption band near 875 nm; RC, reaction center; BChl, bacteriochlorophyll.

ciency of excitation energy transfer from carotenoid molecules to BChl molecules varies for complex type and bacteria species [7]. Up to now the mechanism of excitation energy transfer between carotenoids and BChls has been discussed. Photophysical properties of carotenoid molecules are unusual enough. For example, the band with three peaks within 400–500 nm region in absorption spectrum is attributed to $S_0 \longrightarrow S_2$ transition [8]. The transition $S_0 \longrightarrow S_1$ is symmetry forbidden for carotenoid molecules with conjugation length longer than 7–9, and corresponding band is absent in absorption spectrum. However this is not valid for internal conversion from S_2 to S_1 state, for intermolecular excitation energy transfer, and two-photon excitation of S_1 state [8].

Several pathways of excitation energy transfer from carotenoid molecules to BChls in LH2 complex are possible [9, 10]. First one is via internal conversion from S_2 to S_1 in carotenoid molecule and then intermolecular transfer from lowest singlet excited state S_1 of carotenoid molecule to Q_y level of BChl molecules absorbing within 800–900 nm spectral range. To estimate possibility and effectiveness of this pathway one needs to know spectral position of forbidden $S_0 \longrightarrow S_1$ transition of carotenoid molecule. Several methods how to find spectral position of this transition were proposed [11–13]. The most elegant seemed to be a following one. Two-photon excitation is used for population of S_1 level of carotenoid molecules. Then excitation energy is transferred from carotenoid S_1 level to bacteriochlorophyll S_1 level (Q_y-band), and BChl fluorescence is recorded as a function of two-photon excitation wavelength. The band within 1200 and 1600 nm excitation range (corresponding to 600–800 nm for one-photon excitation), which is not coincide with Q_x and Q_y bands of BChl, is a candidate for $S_0 \longrightarrow S_1$ transition of carotenoid molecules. Such measurements were carried out with several photosynthetic samples (light-harvesting complex LH2 from purple bacteria [9, 14], light-harvesting complex LHC II [10], preparations of photosystem I [15, 16]).

Mutant strains of purple bacteria without carotenoids are known. However, peripheral light-harvesting complex LH2 is not assembling in the cells of carotenoidless mutants of purple bacteria [17]. That is why this complex cannot be isolated from carotenoidless mutants. The use of carotenogenesis inhibitor (diphenylamine) is the only possible way to obtain carotenoidless preparations of LH2. Light-harvesting complexes from the *Alc. minutissimum* cells grown with diphenylamine contain only trace quantities of carotenoid molecules (3–5% of total amount in wild type cells) [18, 19].

LH2 preparations form wild type cells *Alc. minutissimum* and from cells with depleted carotenoid synthesis are useful to test carotenoids role in photosynthetic apparatus via parallel measurements at these preparations. Thus we have shown that dependence of fluorescence intensity on wavelength for two-photon excitation within 1200–1490 (600–745 nm) range is approximately the same for both types of LH2 preparations [20, 21]. It suggests that carotenoid molecules (primarily rhodopin which presents ~68% of carotenoid pool of this bacterium) either are out of excitation or contribute only slightly to fluorescence excitation spectrum in this region. However, two-photon fluorescence spectra of LH2 complex from *Alc. minutissimum* wild-type cells were significantly different from those spectra of wild-type LH2 complex of other purple bacteria [5, 9, 14]. Also we studied spectral properties and excitation energy transfer between B800 and B850 BChl molecules. It was shown that carotenoids (rhodopin) do not influence either the spectral characteristics of B800 and B850 BChls or the femto–picosecond kinetic curves of excitation decay in the B800 band [22].

In the present work a detailed study of BChl fluorescence in the LH2 preparations from the wild-type *Alc. minutissimum* cells and from carotenoid-depleted cells under two-photon excitation within 1200–1500 nm (600–750 nm) spectral range has been conducted.

EXPERIMENTAL

Cells of bacterium *Alc. minutissimum* (MSU strain) were grown for 3–4 days at 30°C in luminostat. To obtain carotenoid-depleted cells, diphenylamine (12 mg/l) was added to the growth media [18]. Chromatophores were obtained by differential centrifugation after cell ultrasonic disintegration [23]. LH2 complex was purified by polyacrilamide gel electrophoresis according to [23]. The carotenoid composition of LH2 complex from wild type cells is as follows: rhodopin, 68.1%; dedihydrorhodopin, 20.0%; spirilloxantin, 4.6%; anhydrorhodovibrin, 5.0%; lycopene, 2.3%. Carotenoid content in the LH2 complex from carotenoid-depleted cells does not exceed 3–5% of the total carotenoid content in LH2 from the wild-type cells.

Absorption spectra of LH2 complex were carried out using the UV-1601 spectrophotometer (Shimadzu, Japan). Corrected fluorescent emission and excitation spectra were recorded with the Fluorolog FL-112 spectrofluorometer (Jobin-Yvon, France) with the near-infrared sensitive photomultiplier R636-10 (Hamamatsu, Japan).

The block diagram of a measuring part used in this study is shown in Fig. 1. Excitation pulses with tunable wavelengths for two-photon fluorescence experiments were generated by an optical parametric amplifier TOPAS (Light Conversion, Lithuania), pumped by a Ti:S regenerative amplifier Spitfire (Spectra Physics, USA). Pulses from a femtosecond generator Tsunami (Ti:S) (Spectra Physics, USA) with a solid-state laser continuous pump laser Millenia Vs (Spectra Physics, USA) were applied to the regenerative amplifier. Pulse solid-state laser Evolution X (Spectra Physics, USA) was used for the regenerative amplifier pumping. The

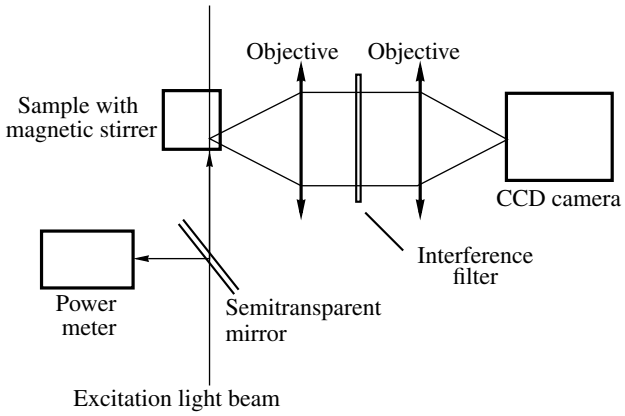

Fig. 1. A schematic diagram of fluorescence registration of light-harvesting complexes from photosynthetic purple bacteria at two-photon excitation by femtosecond laser pulses within near-infrared spectral region (see EXPERIMENTAL).

following light parameters were obtained at the output of the optical parametric amplifier TOPAS: wavelength tuning, within 1200–1500 nm range; pulse duration (FWHM), ~70 fs; pulse repetition rate, 1 kHz; single pulse energy, ~100 μJ.

To separate the signal and idler wavelengths, an output beam of the tunable parametric amplifier was directed to the broadband polarizing cube beamsplitter 05FC16PB.9 (Newport, USA). To dump harmonics under 1200 nm, the interference light-cutting filter FEL1200 (Thorlabs, USA) was used.

Excitation light beam was focused at a sample in a quartz cell by a lens with 8 cm focus length. Some part of light was deflected to a power meter Field Max with detector PS10 (Coherent, USA).

Mean power of light focused at the sample was 0.50 ± 0.04 mW (single pulse energy 500 nJ, pulse repetition rate 1 kHz) during fluorescence excitation spectra measurements. In the curse of measurements constant mean power level of exciting beam was controlled by the home-made automatic control system. This system included round continuously variable metallic neutral density filter NDC-100C-2 (Thorlabs, USA) mounted at the axes of step motor, the power meter Field Max, and personal computer with specially developed LabVIEW program.

Before measurements, concentrated LH2 samples were diluted approximately 15-fold by D_2O buffer (H_2O, in contrast to D_2O, has high absorption within the 1400–1500 nm region and significantly influences excitation intensity within this range). A standard cap-closed 1-cm quartz cell with all polished sides was used for measurements. Optical absorption of LH2 samples from *Alc. minutissimum* at absorption peak near 850 nm was about 5 optical units. To prevent sample degradation by exciting laser pulses a magnetic stirrer inside quartz cell was used.

Fluorescence emission was collected and focused at a detector by means of a condenser consisting of two identical objectives with 17 mm focus length. To separate fluorescence, an interference filter was mounted in parallel with light beam between objectives. The interference filter 10BPF10-900 (Newport, USA) with 60% peak transmission at 900 nm and full wide at half amplitude equal to 10 nm was used. Side by side with this interference filter, a short-pass filter FES1000 (Thorlabs, USA) was placed to dump light with wavelengths >1000 nm to prevent light-striking due to scattered excitation light.

High-sensitive digital CCD camera Matrix-1.4/16U (DeltaTekh, Science park of Moscow State University) was used as a detector. Special LabVIEW program for camera control and data acquisition was developed to take in account all specific requirements of these experiments. Fluorescence light at the camera image looked as an elliptic spot. Its area was not more than ~10% of total image. The program formed two identical square regions at the image before measurement procedure started. One square region overlapped light spot (a fluorescence signal), while the other was placed far from light spot (a background signal). Intensity of fluorescence was defined as a total signal from all elements of a matrix of the CCD camera within the fluorescence rectangular area from which a total signal from all elements within background rectangular area was subtracted. Exposition time for one image was 3 s. Usually each image was measured 5 times. Then a mean intensity of fluorescence was calculated as well as its statistical parameters.

All measurements were carried out at a room temperature.

RESULTS AND DISCUSSION

Absorption spectra of the peripheral light-harvesting complex (LH2) from wild-type cells of *Alc. minutissimum* and from carotenoid-depleted cells are presented in Fig. 2. Of total carotenoid content in LH2 complex from wild-type cells, 68% belongs to rhodopin (conjugation length 11) [19]. Carotenoid absorption band with central peak and two shoulders is located within the 420–570 nm region. Excitation spectrum of BChl fluorescence shows carotenoid band also but its amplitude is lower proportionally to affectivity of excitation energy transfer from carotenoids to BChl. Bands B_x and B_y superimpose and form a common peak near 370 nm ("Sore band"). Band Q_x shows a peak near 590 nm, while Q_y bands of B800 and B850 spectral forms of BChl molecules are near 800 and 850 nm, respectively.

As was shown in [18,19], if diphenylamine (12 mg/l) is added to cell growth media, then carotenoid content of LH2 complex will not be more than 3–5% of its value in the wild-type complex. Besides, rhodopin as a main carotenoid is changed to carotenoid

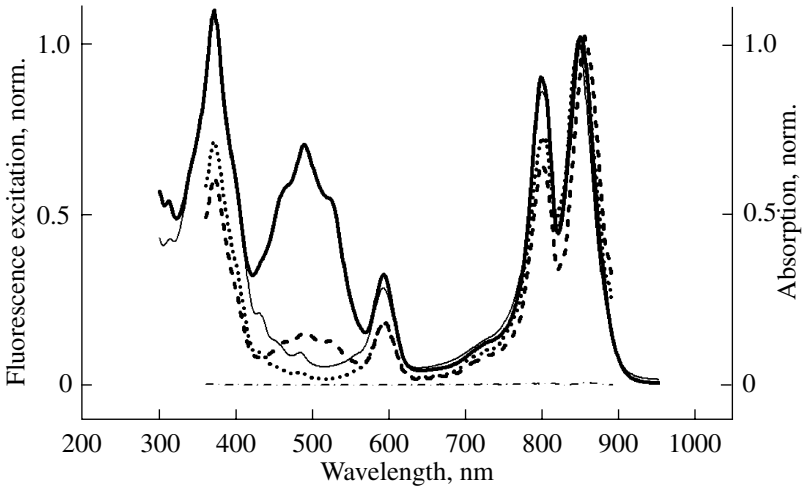

Fig. 2. Absorption spectra of the peripheral light-harvesting complex LH2 from cells of wild-type *Alc. minutissimum* (*solid continuous curve*) and from carotenoid-depleted cells (*thin continuous curve*). Absorption spectra are normalized in a peak of Q_y-band of BChl molecules of spectral form B850 near 850 nm. Fluorescence excitation spectrum of this complex from wild-type cells is shown by dot line, and from carotenoid-depleted cells, by dash line. Fluorescence excitation spectra are normalized to unit in a peak near 850 nm. Fluorescence emission was measured at 930 nm. Fluorescence excitation spectrum of buffer is shown by dot-dash line.

molecules with shorter conjugation length (phytoin, phytofluin, ζ-carotene).

Fluorescence excitation spectra of LH2 complex from *Alc. minutissimum* cells obtained with standard spectrofluorometer are shown also in Fig. 2. Comparison of the absorption spectrum and fluorescence excitation spectrum of the wild-type LH2 complex gives an estimate of about 20% for the effectiveness of excitation energy transfer from carotenoids to BChl. (BChl fluorescence quantum yield of LH2 under excitation to Q_y absorption band near 850 nm is taken to be unity). For LH2 complex from *Rhodopseudomonas acidophila* cells this value is equal to 55% [24] and for LH2 from *Rhodobacter sphaeroides* cells it is near 100% [25].

LH2 fluorescence emission spectrum from wild-type cells corrected to spectral sensitivity of registration system is shown in Fig. 3. Fluorescence peak is near 870 nm and its full width at half amplitude is approximately equal to 50 nm. For LH2 complex from carotenoid-depleted cells fluorescence emission peak is shifted to short-wavelength side by about 7 nm.

Closed circles in Fig. 3 present fluorescence emission spectrum of the LH2 complex from wild-type cells carried out under two-photon excitation by femtosecond pulses with wavelength 1350 nm. The interference filter with transmission peak near 898 nm was used for measurements. Filter rotation by several degrees about light beam axis resulted in short-wavelength shift of transmission peak, transmission decrease, and transmission band broadening. The 30° angle corresponds to a 55-nm shift of transition peak; moreover, the dependence of this shift on the rotation angle is non-linear. However, it is easy to take spectral properties of the filter into account within 0–30° rotation range. Figure 3 shows LH2 fluorescence intensity points under two-photon excitation for different transmission peak wavelengths of the filter within the 898–845 nm range due to filter rotation. One can see that the spectrum of the signal measured under two-photon excitation of LH2 complex coincides with the fluorescence emission spectrum of BChl circular aggregate B850.

The dependences of two-photon signal of LH2 from wild-type cells and from carotenoid depleted cells on mean power of femtosecond pulses with wavelength 1350 nm (675 nm) are presented in Fig. 4. These depen-

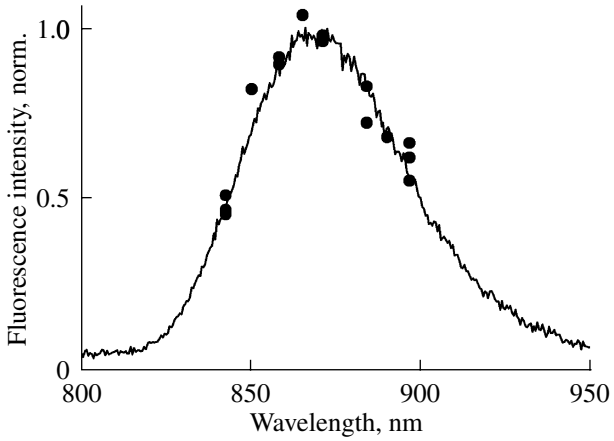

Fig. 3. Fluorescence emission spectrum of complex LH2 from wild-type *Alc. minutissimum* cells (excitation wavelength 380 nm) carried out at standard spectrofluorometer (continuous curve). Fluorescence emission spectrum of the same complex under two-photon excitation at 1350 nm is shown by filled circles (points are measured through the interference filter upon its turn by different angles within 0–30 degrees relative to a direction of the fluorescence beam axis. Mean power of excitation femtosecond pulses was maintained at the level of 0.5 mW.

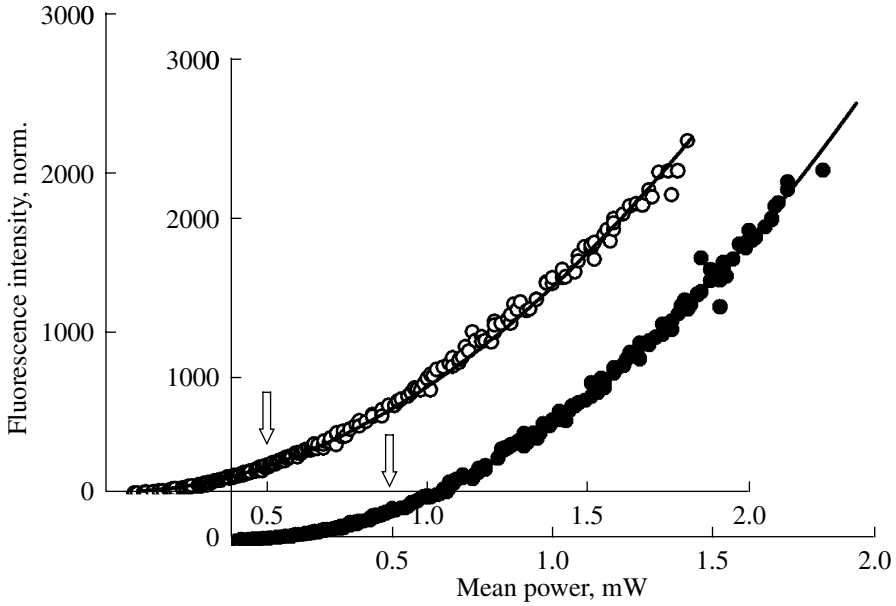

Fig. 4. The dependence of fluorescence intensity of LH2 complex from wild-type cells of *Alc. minutissimum* (closed circles) and from carotenoid-depleted cells (open circles) on excitation energy of femtosecond pulses at 1350 (675) nm. Continuous curves show fitting of experimental points by square dependence on excitation energy. Arrows at curves show excitation energy values corresponding to spectra registration shown in Fig. 5.

dencies were measured within three orders of pulse mean power. It is clear that experimental points can be closely approximated by square dependence on excitation pulse energy (constant background and linear term may be neglected). One can conclude that the measured signal is due to only two-photon excitation.

To measure the dependence of LH2 fluorescence intensity on wavelength of femtosecond excitation pulses (fluorescence two-photon excitation spectrum) mean power of excitation beam was maintained at 0.5 mW level (single pulse energy 500 nJ). This value is shown by arrows at excitation curves (Fig. 4). Fluorescence two-photon excitation spectra for LH2 complex from wild-type cells and from carotenoid-depleted cells measured for 1200–1500 nm spectral region are shown in Fig. 5. These spectra have a form of broad band with maximum near 1340 nm (670 nm, a full width at half magnitude of ~120 nm). Both curves coincide within the accuracy of measurements.

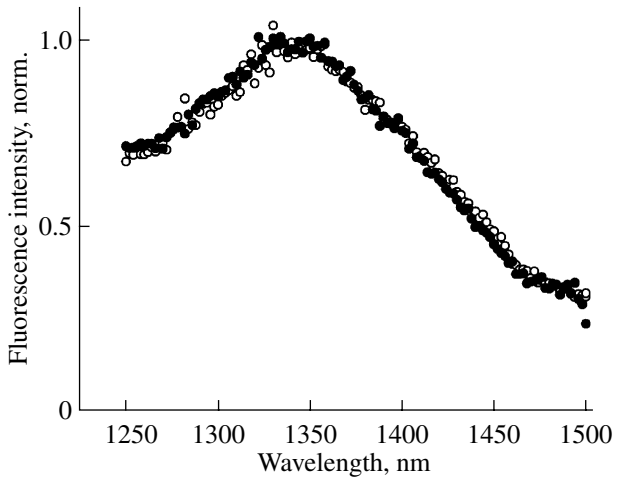

Fig. 5. Two-photon fluorescence excitation spectra of LH2 complex from wild-type cells of *Alc. minutissimum* (closed circles) and from carotenoid-depleted cells (open circles).

As these spectra coincide within the accuracy of measurements, then the broad band with peak near 1350 nm (675 nm) cannot be attributed to optically forbidden transition $S_0 \longrightarrow S_1$ of carotenoids (rhodopin). Possibly, the rhodopin transition $S_0 \longrightarrow S_1$ is somewhere within this spectral region but its absorption cross-section is at least 20-fold lower than its value for the broad band with peak near 1350 nm (675 nm) or else, the effectiveness of excitation energy transfer from first singlet excited state of carotenoids (rhodopin) is very low.

As carotenoids are excluded from consideration as the reason of occurrence of the absorption broad band with a peak near 1350 (675) nm in a spectrum of two-photon excitation of LH2 complex, it is necessary to assume that this band is connected with any BChl molecules of this complex. Fluorescence from the lowest excitonic level of circular aggregate of BChl molecules (spectral form B850) is registered in experiments with two-photon excitation (see Fig. 3). However, it is not obvious that just this level of complex LH2 is excited via two-photon excitation; excitation energy can get on it as a result of intramolecular conversion or intermolecular transfer in a complex. There is no BChl absorp-

tion bands within the 600–800 nm region in absorption spectrum of complex LH2 (see Fig. 2). Hence, the band with peak near 1350 (675) nm is forbidden at one-photon excitation, but is allowed at two-photon excitation. Preliminary pump and probe measurements of photoinduced absorption changes of both types of LH2 complex within the 790–900 nm range under two-photon excitation at wavelength equal to 1350 nm showed that absorption changes are revealed only within the B850 band. Note that absorption changes are induced in both bands (B800 and B850) in the case of one-photon excitation at 800 nm. So, two-photon excitation of LH2 samples results in appearance of excited states only at the BChl molecules of B850 spectral form or, in other words, at BChl molecules of circular aggregate with strong enough excitonic interaction (~300 cm^{-1}) [26]. "Monomer" BChl molecules of B800 spectral form, which are characterized by weak excitonic interaction between each other and with B850 molecules (~30 cm^{-1}), apparently are out of processes of excitation energy absorption and transformation under two-photon excitation. (The detailed data of photoinduced absorption changes of LH2 complex under two-photon excitation will be published elsewhere).

According to calculations for complex LH2 in the framework of the simplified excitonic theory, which does not consider interactions of excitons with phonons under two-photon excitation, only even transitions should be resolved: $k = 0, \pm 2, \ldots$ [27]. However, the region near 675 nm (1350 nm), where the broad band is registered in two-photon excitation spectrum of fluorescence, should correspond to excitation of excitonic bands with great values k, the intensity of which should be low enough. However, probably the resonance enhancement of two-photon excitation of the BChl molecules of circular aggregate may occur just in this spectral region [28, 29].

According to another possible explanation, the wide band in the spectrum of two-photon excitation of complex LH2 can be caused by two-photon excitation of the optically forbidden level(s) of the charge-transfer state(s) of circular aggregate (or BChl dimers of circular aggregate) [30].

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