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Transient Absorption Study of Two-Photon Excitation Mechanism in the LH2 Complex from Purple Bacterium *Rhodobacter sphaeroides*

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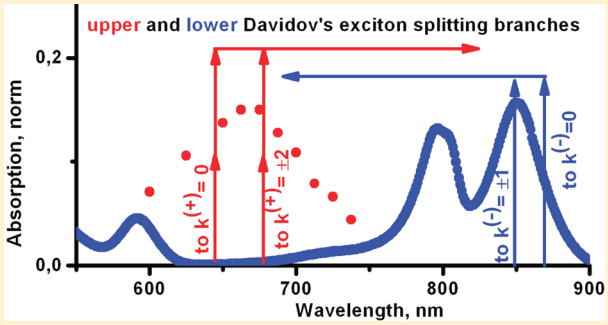
ABSTRACT: The mechanism of two-photon excitation of a peripheral light-harvesting complex LH2 (B800−850) from purple bacterium *Rhodobacter sphaeroides* was explained on the basis of femtosecond transient absorption data. Fast bleaching of the B850 absorption band was measured under two-photon excitation by 1350 nm femtosecond pulses, showing fast subpicosecond arrival of excitation energy to B850 circular aggregates. Any spectral changes connected with the B800 absorption band of B800-BChl molecules were absent. A similar picture was observed under one-photon excitation of the LH2 complex by 675 nm femtosecond pulses. We believe these effects may be attributed to direct excitation of high-energy excitonic states of a B850 circular aggregate or its vibrational manifold in accordance with the model of Abe [*Chem. Phys.* **2001**, *264*, 355−363].



INTRODUCTION

In the photosynthetic apparatus of purple bacteria, each photoactive reaction center (RC) is surrounded by a "core" light-harvesting complex (LH1). In addition to the complex LH1, many bacteria have two to six copies of a "peripheral" light-harvesting complex (LH2) per one RC. The light-harvesting complexes of purple bacteria contain two types of pigment molecules: the main pigment (bacteriochlorophyll − BChl) and auxiliary pigments (carotenoids − Cars). Photophysical properties of these pigments by themselves and in the light-harvesting complexes are rather unusual and remain the subject of intense research.

In 1982 we revealed a number of unusual properties of the BChl long-wavelength absorption band (Q_y band) of light harvesting antenna of purple bacteria,[1,2] which later led us to the assumption that BChl molecules in the antenna form a symmetrical circular aggregate showing a strong exciton interaction.[3] This model is in accordance with the optical properties of light-harvesting antenna of purple bacteria, in particular the data of pico- and femtosecond laser absorption spectroscopy[4] and hole-burning experiments.[5] Subsequent X-ray studies of light-harvesting complexes LH2[6,7] and LH1[8] confirmed the arrangement of the BChl molecules in symmetrical circular excitonically coupled aggregates in these complexes. Notably, the refinement of BChl positions according to X-ray data is consistent with the results of previous calculations of the optical properties of complex LH2.[9]

While describing the optical properties of light-harvesting complexes, one must take into account the excitonic nature of the BChl aggregates, which determines its energy state structure. In the case of a B850 ring with 18 BChl molecules, there are two exciton level sets (two Davydov components each of nine states). The linear absorption spectrum of the lower (red-shifted) set consists of a strong band corresponding to the degenerated state $k = \pm1^{(-)}$ and a weak long-wavelength line corresponding to the $k = 0^{(-)}$ state. The exciton levels of the upper (blue-shifted) set give no visible contribution to linear absorption (a line corresponding to the $k = 0^{(+)}$ state is too weak to be seen in the linear absorption spectrum).

The position of the lowest $k = 0^{(-)}$ exciton state has been found using hole-burning spectroscopy, being at 865 nm or ∼11550 cm^{-1}.[10,11] However, the location of the high-energy state $k = 0^{(+)}$ has not been reliably determined. A weak band at 780 nm (∼12800 cm^{-1}) in the circular dichroism spectrum was ascribed to it in ref 12. Later, however, this feature was assigned to vibronic manifold of free BChl molecules.[13] Using polarized fluorescence excitation spectroscopy and model simulations, the value of 760 nm (∼13200 cm^{-1}) was determined in 14. It is suggested that the upper edge of exciton bandwidth can be detected by reflection spectroscopy[15] or two-photon spectroscopy.[16] According to ref 16, in a symmetrical circular aggregate (B850), the upper dipole-forbidden state $k = 0^{(+)}$ turns out to be two-photon-allowed and may be investigated by two-photon absorption or excitation spectroscopy.

The value of the $k = 0^{(+)}$ state energy is important for determining the exciton coupling energy in B850 aggregate of

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BChl molecules. The exciton coupling energy can be roughly estimated as a quarter of the exciton spectral bandwidth, i.e., the range between $k = 0^{(-)}$ and $k = 0^{(+)}$ states. For now, a consensus is still lacking about the value of the coupling energy. Both experimental and theoretical arguments have been raised supporting coupling energies between 200 and 800 cm^{-1}.[17]

Carotenoids (auxiliary pigments) play an important role in photosynthesis too. Carotenoids act as integral structural components, quench BChl triplet states to prevent formation of singlet oxygen, harvest light quanta, and efficiently transfer excitation energy to nearby BChl molecules.[18,19] Strong absorption of carotenoids in the visible spectral region is the result of an electronic transition from the ground state to the higher singlet state, S_2. The first excited singlet state, S_1, of carotenoids is optically "dark", that is, one-photon $S_0 \rightarrow S_1$ transition is forbidden by the selection rules.[20] However the selective excitation of the carotenoid S_1 state can be achieved by two-photon excitation because transitions between states of symmetry Ag, such as S_0 and S_1, are two-photon-allowed.[21]

Taking into account carotenoid-to-BChl excitation energy transfer (EET) in LH2 complexes, two-photon-sensitized fluorescence of BChl may be used as a probe for two-photon excitation of carotenoids. This approach has been used recently to clarify the properties of carotenoid excited state processes in bacterial as well as in higher plant pigment−protein complexes. The two-photon excitation spectra of BChl fluorescence, for example, in the range of 1100−1500 nm for peripheral purple bacterial light-harvesting complex (LH2) of *Rhodobacter sphaeroides*[22,23] and in the range of 1100−1400 nm for the higher plant antenna complexes, LHC II[24] and PS-I,[25,26] revealed broad bands near 650 and 600 nm, respectively, that were assigned to the carotenoid S_1 state. The occurrence of these bands was taken as evidence for the participation of carotenoid S_1 states in EET processes. For the development of concepts of EET between carotenoids and chlorophylls, see the recent papers and references therein.[27,28]

A clear way to distinguish the role of carotenoids in EET processes is a parallel investigation of carotenoid-containing and -depleted complexes.[29] Recently we applied two-photon fluorescence excitation spectroscopy to LH2 complexes from purple bacteria *Allochromatium minutissimum* and *Rhodobacter sphaeroides* within 1200−1500 (600−750) nm spectral range.[30,31] It was shown that there is no difference between the two-photon fluorescence excitation spectra of LH2 complexes from *All. minutissimum* wild-type cells (containing carotenoids) and from cells with suppressed carotenoid synthesis. A similar result was obtained in parallel measurements on the LH2 complex from *Rb. sphaeroides* wild-type cells and pseudo-LH2 complex from carotenoidless cells of mutant R-26 of this bacterium. According to our results, two-photon excitation of complexes LH2 in that spectral region is not carotenoid-mediated.

In this paper we used femtosecond transient absorption spectroscopy to determine the target of two-photon excitation within the LH2 complex. It is shown that two-photon (at 1350 nm) and one-photon (at 675 nm) excitation of the complex LH2 nm can be attributed to direct excitation of high-energy excitonic state of B850 circular aggregate or its vibrational manifold.

EXPERIMENTAL METHODS

Rb. sphaeroides cells were grown in Hunter's medium. Grown cells were suspended in 0.01 M.Tris-HCL buffer (pH 8.0) and disrupted by ultrasonic apparatus UZG-01.10 ("Ultra-Filter", Russian Federation) during 3 min procedure. Crude chromatophore fraction was obtained as supernatant after the deposition of whole cells and large fragments in the 20 000 g. To isolate the light-harvesting complex LH2 of *Rb. sphaeroides,* the fraction was diluted to $A_{850} = 50$ cm^{-1} in 0.01 M Tris-HCl buffer (pH 8.0) with the addition of 20% *n*-dodecyl-*β*-D-maltoside to a final concentration of 1.5%. Incubation of detergent was performed at 24 °C in the dark with constant stirring for 45 min. After that, nonsolubilized material was removed by centrifugation at 20 000*g* for 10 min. Supernatant was layered on a step gradient of sucrose (0.3M/0.6M/1.2M) with 0.2% *n*-dodecyl-*β*-D-maltoside and centrifuged for 3 h at 200 000*g*. The fraction of light-harvesting complex was localized in 1.2 M sucrose. It was collected and concentrated on the tubes Amicon Ultra 15 and density of about 200−400 optical units at 850 nm.

The sample was diluted to optical density ∼0.3 OD in the 2 mm flow cell at the wavelength of 850 nm (∼10^{15} LH2 complexes per cm^3 in solution). To elucidate the effect of strong water absorption in the 1400−1500 nm region, the samples were diluted with D_2O-based buffer. During transient absorption measurements, the sample was pumped through a 2 mm quartz flow cell.

Transient absorption spectroscopy measurements were done using a spectrometer based on optical parametric amplifier (OPA) TOPAS, pumped at 790 nm by a Ti:Sa regenerative amplifier Spitfire (Spectra Physics, Mountain View, CA). Pulses from OPA with 1 kHz repetition rate and tunable wavelength were used for sample excitation. Part of light from regenerative amplifier was also focused info sapphire plate to generate white-light continuum used for probing absorption changes. In addition to ordinary probe beam, a probe-reference beam was used, which passed through the sample outside the area of excitation. The deltaOD spectra were recorded as deltaOD = $(I_{probe}/I_{ref})_{w/o\_excitation}/(I_{probe}/I_{ref})_{on\_excitation}$. The polarization between pump and probe pulses was set to a magic angle (54.7°). The duration of pump pulse was about 70 fs and remained constant over the entire wavelength range. Pulse energy for the two-photon excitation was set to 300 nJ (peak intensity 1200 MW/cm^2, or about 10^{15} photons/cm^2/pulse); for one-photon excitation, it was ser to 10 nJ (peak intensity 40 MW/cm^2, or about 10^{13} photons/cm^2/pulse). These pulse energies resulted in bleaching of about 0.5×10^{-3} OD at 850 nm for both one-photon and two-photon experiments.

For basic absorption measurements, we used a UV-1601 spectrometer (Schimadzu, Japan). After each transient absorption measurement, the absorption spectrum was recorded to check that there was no degradation of the sample.

RESULTS AND DISCUSSION

In previous papers, the two-photon excitation spectra were recorded by measuring the fluorescence intensity of the BChl long-wavelength absorption band (B850). In this paper we applied femtosecond transient absorption spectroscopy to follow excitation dynamics in the LH2 complex from purple bacterium *Rb. sphaeroides*.

The rate of energy transfer after the two-photon excitation was monitored by time-dependent bleaching of the Q_y absorption band at 850 nm after the excitation. Delta absorption spectra at two delay times obtained after two-photon excitation at 1350 nm are shown in Figure 1, and the corresponding kinetic trace is shown in Figure 2 (trace 1). The

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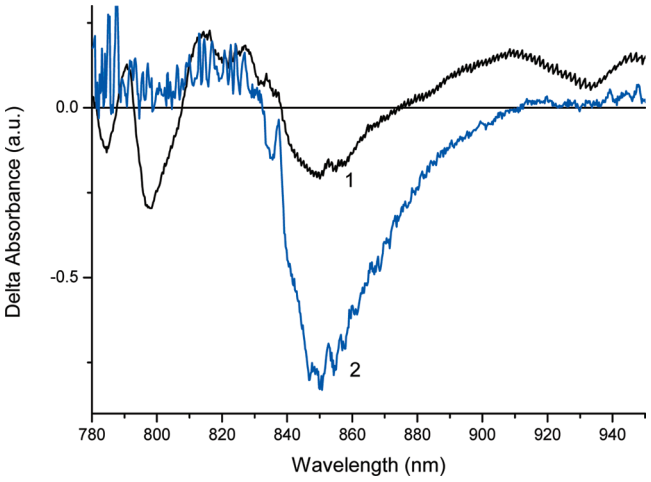

Figure 1. Observed difference spectra at 30-fs (1) and 100-fs (2) delays between pump and probe pulse under two-photon excitation at 1350 nm.

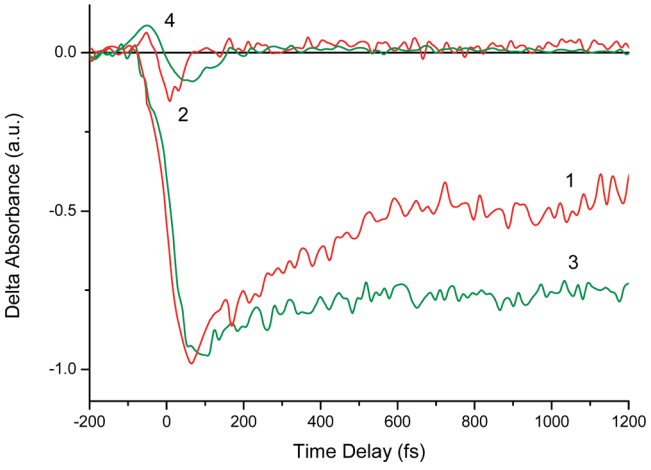

Figure 2. Observed kinetic traces after two-photon excitation at 1350 nm followed by probe at 850 nm (trace 1) and 800 nm (trace 2), and after excitation at 675 nm followed by probe at 850 nm (trace 3) and 800 nm (trace 4).

rise of B850 bleaching is characterized by the instantaneous (pulse duration limited) component, which corresponds to fast (within ~100 fs) arrival of excitation energy to B850 molecules. After the rise, a long-lived kinetic component is seen corresponding to the slow (within several hundreds of picoseconds) decay of the remaining excitation of B850.

The early time spectrum taken at 30 fs shows induced absorption in the region above 880 nm. Later this absorption is replaced by bleaching in that spectral region corresponding to stimulated emission from B850. Also, a slight time-dependent spectral shift of the delta absorption spectrum can be noticed within first 100 fs after excitation. During the decay after the final buildup occurring at 100 fs, no noticeable changes in the shape of the δ absorption spectra were detected. The features described above reflect the energy relaxation processes within B850 circular aggregate, which by our estimation occur within first 100 fs after excitation.

The B800 band bleaching is absent after two-photon excitation at 1350 nm (see trace 2 in Figure 2). However, it is well-known that excitation of B800 molecules (direct, via EET or via relaxation) results in short-lived bleaching ($\tau \sim 0.7$ ps at room temperature) at 800 nm due to fast energy transfer from B800 molecules to B850 molecules of circular aggregate.[32−34] Thus two-photon excitation at 1350 nm leads to selective excitation of BChl molecules of circular aggregate B850.

The two-photon excitation spectrum measured by altering excitation wavelength within the range 1200−1500 nm and probing residual Q_y bleaching at 10 ps delay between pump and probe pulse is shown in Figure 3 (triangles). The spectrum is

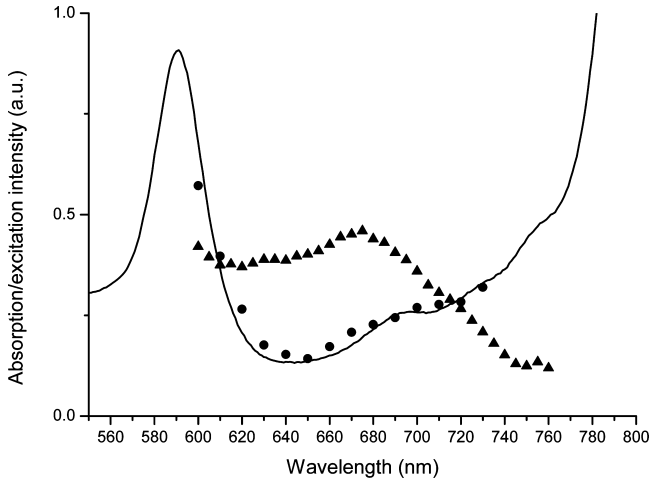

Figure 3. One-photon (circles) and two-photon (triangles) excitation spectra of LH2 isolated from wild-type cells of *Rb. sphaeroides*. Linear absorption spectrum (solid line) is shown for comparison.

pretty much similar to the one obtained by probing the fluorescence intensity of B850 in our previous papers.[30,31] It has a wide excitation band around 1350 nm (14 800 cm^{-1}). Earlier we showed that this band is a result of two-photon excitation, but is not carotenoid-mediated.[30,31] Therefore, for this investigation we used only LH2 complex from *Rb. sphaeroides* wild-type cells (carotenoid-containing). LH2 preparations from carotenoid-depleted or from carotenoidless mutant cells were not used in this work.

For comparison, we measured the one-photon excitation spectrum in the corresponding one-photon spectral range 600−750 nm using the same setup and approach. As in the normal linear absorption spectrum, no pronounced bands can be seen in the one-photon excitation spectrum around 675 nm (Figure 3, circles), although the excitation spectrum is clearly nonzero over the entire measurement range. Also, it turns out that the direct one-photon excitation at 675 nm leads to exactly the same transient absorption picture as the two-photon excitation at 1350 nm (Figure 4). That includes the absence of B800 excitation and the features reflecting relaxation dynamics (see above). The direct one-photon excitation at 685 nm followed by fast relaxation (less than ~100 fs) has been previously reported for B850 complex from *Rhodopseudomonas acidophila* (see ref 35). This effect was preliminarily assigned to excitation of B850 high-energy vibronic states, without further details.[35]

Summarizing the above, we conclude that both one-photon and two-photon excitations lead to fast selective excitation of B850 circular aggregate of LH2 complex. We believe that as a first approximation, these effects can be explained by a simple model of one- and two-photon spectra of symmetric aggregates of asymmetric molecules described in ref 16. According to this model for the dimer with an antiparallel direction of transition

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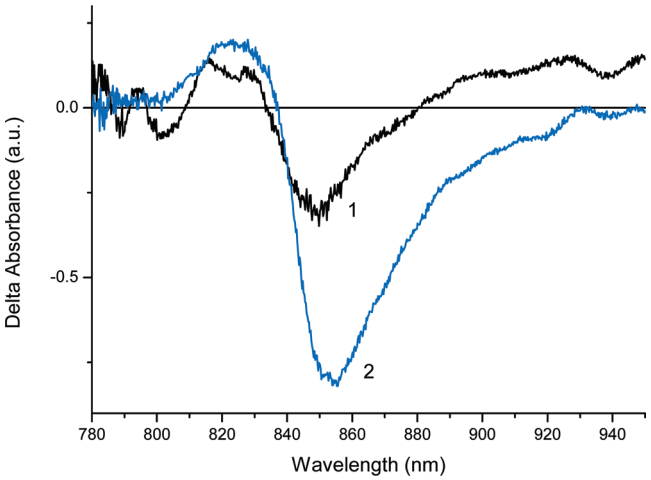

Figure 4. Observed difference spectra at 30-fs (1) and 100-fs (2) delays between pump and probe pulses under one-photon excitation at 675 nm.

dipoles, one-photon absorption is allowed for the transition to the lower exciton level and forbidden for that to the higher level. For two-photon absorption, the transition is allowed only to the upper exciton level. A similar situation is obtained for the LH2 complex,[16] in which dipole transitions of each pair of B850 BChl molecules are quasi-antiparallel. The most long-wavelength levels $k = 0^{(-)}$ and $k = \pm 1^{(-)}$ of the lower$^{(-)}$ Davidov exciton branch are allowed for one-photon absorption. For two-photon absorption, only even levels $k = 0^{(+)}$ and $k = \pm 2^{(+)}$ are allowed belonging to the upper$^{(+)}$ branch of exciton. It should be noted that in the case of quasi-antiparallel dipoles of BChl pairs of B850 aggregate, the exciton band $k = 0^{(-)}$ of the lower branch$^{(-)}$ has the longest wavelength position, and $k = \pm 1^{(-)}$ bands are shifted toward shorter wavelengths. Conversely, the exciton band $k = 0^{(+)}$ of the upper branch$^{(+)}$ has the shortest wavelength position, and $k^{(+)} = \pm 2$ bands are shifted to longer wavelengths with respect to it. The two-photon absorption is dominated by the states with $k^{(+)} = 0$ and $k^{(+)} = \pm 2$. Contributions from all other states are orders of magnitude smaller. That is why the spectrum of two-photon absorption of the B850 aggregate of LH2 complex is significantly shifted toward shorter wavelengths relative to the corresponding spectrum of single-photon absorption.

According to this simple qualitative model, the spectral position of the two-photon excitation band suggests that the upper excitonic state $k = 0^{(+)}$ has energy of about 14800 cm^{-1}, and the exciton bandwidth is about 3200 cm^{-1}. Thus, the exciton coupling energy can be roughly estimated as 800 cm^{-1}, a quarter of exciton bandwidth. This value is more than twice the generally accepted value ~300−400 cm^{-1} (see, for example, ref 36). It should be noted that the value of 300−400 cm^{-1} is not experimentally measured. It was obtained as a parameter in the theoretical approximation of the spectral and kinetic curves of the LH2 complex.

Our experimental data are in reasonable accordance with calculations in ref 16. Under conditions of two-photon excitation (see Experimental Methods), the measured bleaching at 850 nm was 0.5×10^{-3} OD, which means that about 1 of 1000 LH2 complexes is excited under absorption of two photons. Thus we can estimate the two-photon absorption cross-section as ~10^{-27} cm^4 W^{-1} or ~10^4 GM. A value of the same order is reported in ref 16 as a result of model calculations. Similarly, the one-photon absorption cross-section can be estimated as ~10^{-16} cm^2, which is also in accordance with model calculations.

However, it is unlikely that the spectral position of the two-photon excitation band at about 1350 (675) nm directly corresponds to upper excitonic states $k^{(+)} = 0$ and $k^{(+)} = \pm 2$. Quantitative modeling of the B800−B850 spectra using the disordered exciton model suggested the upper exciton states of the B850 ring in the 770−815 nm region.[37] Measurements of the CD spectrum[38] showed the 780 nm peak originating from the upper exciton component of the B850 excitonic manifold. These estimates do not support the idea of a pure excitonic origin of the observed 675 nm band. Moreover, the observed band is too broad (640−720 nm, i.e., 1700 cm^{-1}) compared to what we could expect for the purely excitonic band (i.e., 470 cm^{-1} full width at half-maximum (fwhm) of the 780−810 nm upper Davydov component). Such a broad and blue-shifted band can be determined by the charge transfer (CT) states with high energies that are coupled to the upper exciton transitions. In two-photon experiments these CT states can be reached via upper exciton states. The probability of such a process is given by the product of the transition dipole for the exciton state and the static dipole for the CT state involved. Notice in this respect that the existence of the CT states coupled to the excited states of the tightly packed B850 ring have been considered in literature. It was suggested that such a coupling is responsible for significant (4 times) difference in the static and dynamic disorder values for the B850 nm and B800 nm bands.[37] Similarly, one could suppose the existence of some CT state (or manifold of the CT states) near 675 nm with anomalously big homogeneous (phonon-induced) and inhomogeneous (disorder-induced) broadening (typical for the CT states). Weak coupling to the upper exciton states (that are weakly allowed themselves) makes these CT states invisible in linear absorption, but they can be viewed by two-photon technique.

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