Excited-state dynamics of mutated antenna complexes of purple bacteria studied by hole-burning

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Absorption and fluorescence excitation spectra of various LH2 antenna complexes of two purple bacteria at low temperature (1.2 and 4.2 K) have been measured, and energy transfer rates within these complexes have been determined by spectra hole-burning. The systems studied were membranes of a wild-type strain of Rhodobacter sphaeroides, membrane samples from four LH2-only strains containing specifically mutated LH2 complexes of the same bacterium, and the isolated B800–820 complex of Rhodopseudomonas acidophila (strain 7050). The mutants exhibit blue-shifted B850 absorption bands with their spectral positions depending on the specific amino acid residues replaced in the α-polypeptide sequence. Energy transfer rates from B800 to B850 (or to their respectively blue-shifted bands) have been obtained by hole-burning experiments in the B800 band. The mutants of Rb. sphaeroides and the LH2 complex of Rps. acidophila yielded transfer times similar to those of the B800–850 complex of Rb. sphaeroides. These values, which for the various complexes vary between 1.7 and 2.5 ps in the wavelength region from 798 to 805 nm, do not decrease monotonically with the spectral distance between the bands. Various models based on Förster's energy transfer mechanism are discussed, of which only one is consistent with the results. In this model the energy is assumed to be transferred not directly from the Q O-O band of B800 to that of the (blue-shifted) B850, but indirectly through the excitation of a vibrational mode.

1. Introduction

The primary step in bacterial photosynthesis is the absorption of light and the transfer of excitation energy from the antenna pigment–protein complexes to the reaction center (RC), where the charge separation occurs. These energy transfer and trapping processes take less than 100 ps. In the intracytoplasmic membrane of the purple bacterium Rhodo bacter sphaeroides there are two types of light-harvesting (LH) or antenna complexes; the B800–850 (also called LH2) and the B875 (or LH1) [1]. In Rhodopseudomonas acidophila (strain 7050), on the other hand, there are three LH complexes, of which two are peripheral, the B800–820 and the B800–850, and one is a core antenna, the B875 [2]. While the LH1 complex lies in the direct neighbourhood of the reaction center, the LH2 complex is peripheral and in contact only with LH1 [1,3]. The energy is thus transferred from LH2 to LH1, and from here to the RC complex.

The antenna pigments, bacteriochlorophyll a (BChl a) molecules, are non-covalently bound to two small transmembrane α- and β-proteins of 50–60 amino acid residues each [3,4]. The central part of the membrane is hydrophobic and assumed to have an α-helical conformation. The binding of the BChl a molecules, at least for BChl 850 in the LH2 complex, is thought to occur via the central Mg atom to the histidine residues located in the hydrophobic domain [3]. While the BChl 850 molecules appear to have their molecular plane perpendicular to the membrane, the BChl 800 lie approximately in the membrane plane [5]. The α- and β-polypeptides of
the individual complexes in the various purple bacteria have a relatively high sequence homology [3,4]. Aromatic amino acid residues placed in the vicinity of the pigment binding sites are thought to have structural and/or functional effects [3] and to be responsible for specific properties of pigment–protein complexes [3,4,6].

It was recently shown by site-directed mutagenesis that the spectral properties are indeed influenced by these amino acid residues [7]. By replacing the two tyrosines (Y) by phenylalanine (F) and leucine (L) at positions 44 and 45 of the α-polypeptide chain in the LH2 complex of Rb. sphaeroides, the absorption band at 850 nm is shifted to the blue. If only one of the two (YY) residues is replaced by phenylalanine (F), a mutant (FY) results, which has a band at 839 nm at 77 K. If both tyrosines are replaced, the B850 band shifts to \( \approx 829 \) nm (FL) at 77 K and a spectrum similar to that of the isolated B800–820 complex of Rps. acidophila is obtained [7]. In all these mutants the spectral position of the B800 band remains unchanged, probably because these residues do not interact with the BChl 800 molecules but only with the spatially closer BChl 850.

A crucial question which arises from the results of ref. [7] is whether the energy transfer rates from B800 to the blue-shifted B850 bands in these mutated LH2 complexes are affected, and if so, to what extent. We would, in principle, expect a change if Förster’s classical energy transfer mechanism [8] is valid because in his model a greater spectral overlap between the bands of the fluorescence spectrum of B800 and those of the absorption spectrum of the blue-shifted B850 would lead to a higher rate. In the present work we have addressed this question by spectral hole-burning at low temperatures.

Thus far, energy transfer rates have been determined in intact purple bacteria and isolated complexes without amino acid residue mutations. In particular, the B800→B850 energy transfer time in the LH2 complex of Rb. sphaeroides has been reported to be \( \approx 1–2 \) ps by picosecond fluorescence and polarized absorption measurements at 293 K [2,9–11] and at 77 K [10–12]; sub-picosecond transient absorption experiments on the same system at room temperature yielded a B800→B850 transfer time of 0.7 ps [13,14]. Upon lowering the temperature the spectral bandwidths become narrower and less overlapped. We have previously reported a spectral hole-burning experiment in which we found that the B800 absorption band of the B800–850 complex of Rb. sphaeroides is inhomogeneously broadened at liquid helium temperature, and the B800→B850 energy transfer time is 2.3±0.4 ps, independent of temperature between 1.2 and 30 K [15]. This value was subsequently confirmed at 1.6 K, again by hole-burning [16].

Only a few energy transfer experiments, so far, have been reported for the LH2 complexes of Rps. acidophila [2,17,18]. From picosecond absorption spectroscopy at 800 nm with 10 ps laser pulses [2] the energy transfer from B800 to B820 at room temperature was too fast to be detected (<0.5 ps), whereas at 77 K the rate had slowed down sufficiently to be observable, but the transfer time still was 0.5–1 ps [2,11]. These times are significantly shorter than those reported for the B800→B850 transfer in Rb. sphaeroides [10–12]. The difference was attributed to the larger spectral overlap of the B800 and B820 bands in Rps. acidophila, from which the rate of Förster energy transfer was estimated to be at least a factor of three higher than that for the B800→B850 process in Rb. sphaeroides [2]. Also the apparently much stronger temperature dependence of the B800→B820 transfer time was attributed to a temperature-induced change in the spectral overlap [2]. From the measured times, and assuming a Förster mechanism, a distance of \( \approx 20 \) Å was calculated between BChl 800 and BChl 820 molecules, and of \( \approx 15 \) Å between identical BChl 850 or identical BChl 820 chromophores [2,11]. Very recently, hole-burning experiments on another complex of Rps. acidophila at 4.2 K, the B800–850, were reported [18]. From the widths of the holes burnt in the B800 band, which were found to be independent of wavelength between 794 and 819 nm, it was concluded that the B800* lifetime is 1.8±0.2 ps, somewhat longer than measured by picosecond spectroscopy at 77 K for the B800–820 complex [2,11]. The results also suggested that the B800→B800 energy transfer is significantly slower than the B800→B850 transfer [18].

If the only parameter that determines the B800→B850 (or B820) energy transfer rates in LH2 complexes were the spectral overlap of the Q\(_{\pi}\), 0–0 B800 emission band with the Q\(_{\pi}\), 0–0 B850 absorption band (which is blue-shifted in the mutants of...
Rb. sphaeroides), as often assumed, one would expect a strong monotonic increase of the rate by about five to six orders of magnitude with the inverse of the separation of these bands. Such an increase has not been observed in the present hole-burning experiments in the B800 bands of four mutants of Rb. sphaeroides and of the B800–820 complex of Rps. acidophila at 1.2 and 4.2 K, see section 3.2. The energy transfer times from the BChl 800 to the (blue-shifted) BChl 850 molecules in the mutants and in Rps. acidophila are compared to those previously reported by us for the intact membranes and the isolated B800–850 complex of Rb. sphaeroides [15] and to values in the literature [16,18,19]. Various models based on Förster’s weak coupling energy transfer mechanism [8] are tested, of which only one is able to explain the experimental results here obtained.

2. Experimental

2.1. Samples

Five membrane preparations from various strains of Rb. sphaeroides were studied together with the isolated B800–820 LH2 complex of Rps. acidophila (strain 7050). The membranes from the wild-type strain 2.4.1. of Rb. sphaeroides contain LH2, LH1 and reaction center complexes. Since the LH2 antenna complex is the object of study here, these wild-type membranes will be called WT (YY), where the two letters “Y” and for the two tyrosine residues at positions 44 and 45 in the original α-polypeptide sequence of LH2. The mutant designated B800–850 (YY, “control”), formerly called “pseudo” wild type in ref. [7], was prepared from a strain of Rb. sphaeroides that had been depleted of the genes encoding the polypeptide components of the LH2, LH1 and RC complexes, and in which only the original LH2 genes were re-inserted [20]. Thus, the measurements could be carried out with the LH2 antenna complex in a membrane environment free from interference from LH1 and the RC. In a similar fashion, the LH2-only mutants B800–841 (FY) and B800–839 (YF) were obtained following site-directed mutagenesis of the LH2 genes [7,21]. In these cases one of the tyrosine (Y) residues was replaced by phenylalanine (F) at positions 44 and 45, respectively. When both tyrosine (YY) residues are replaced by phenylalanine (F) and leucine (L) a fourth mutant denoted by B800–830 (FL) is obtained [7].

The membranes of the four LH2-only mutants of Rb. sphaeroides were prepared by disrupting the cells with a French press [22], whereas the WT (YY) membranes were prepared from cells by sonication and diluted in a buffer solution containing 20 mM tris–HCl (pH 8). The B800–850 complex was isolated from wild-type membranes using the detergent lauryldimethylamine N-oxide (LDAO) following the method of Frank et al. [23]. The preparation was done in the dark at 4°C. Concentrated solutions of the isolated complex were diluted with 20 mM tris–HCl buffer (pH 8) and 0.1% LDAO in water. The B800–820 complex of Rps. acidophila (strain 7050) was prepared according to ref. [24]. The solutions contained a buffer of 10 mM HEPES (pH 8) and 0.1% LDAO in water. In order to obtain glassy samples of good optical quality at low temperature, the solutions of all samples were diluted with glycerol in a ratio of about 60%–75% (v/v) glycerol: 40%–25% buffer/LDAO/water [15]. The optical densities of 3.5 mm thick samples at the maximum of the 800 nm bands at room temperature varied between 0.25 and 1.0. The samples were stored in the dark at −20°C before use. Cooling of the samples to liquid helium temperature was performed in less than one minute, in the dark.

2.2. Low-temperature (4.2 K) spectroscopy and hole-burning

Broad-band absorption spectra at 4.2 K were recorded by focusing the light of a halogen lamp, passed through a water filter to remove the infrared part of the spectrum, on the sample and detecting the transmitted light through a 0.85 m double-monochromator (Spex 1402, resolution 2 Å) with either a liquid N2-cooled photomultiplier (EMI 9684) or with a photodiode (EG&G, model HUV-4000), in combination with a lock-in amplifier (EG&G, Brookdeal, model 9503) and a chopper (HMS, model 221). The intensity of the signal at each wavelength was subsequently divided by that of a reference spectrum of the same buffer solution, and the logarithm of the ratio was plotted. Different samples from the same original batch, stored in the dark at −20°C,
were used for the spectra and the hole-burning (HB) experiments.

A titanium:sapphire laser (Coherent 899-21, amplitude stabilized to <0.5% by an electro-optic modulator) pumped by an Ar+ laser was used without the intra-cavity assembly to perform HB experiments in the 800 nm bands of all LH2 complexes of \textit{Rb. sphaeroides} and \textit{Rps. acidophila}. The laser bandwidth was about $6 \pm 2$ GHz. Its wavelength was calibrated with a Michelson-type interferometer wave-meter and scanned with a linear actuator (Oriel, Encoder Mike 18246), controlled by a PC (the smallest step of the actuator was $\approx 5$ GHz).

The laser beam was focused on the sample (spot size, $A \approx 6-10 \text{ mm}^2$), and burning-power densities ranging from $P_{\text{b}}/A = 90$ to $900 \text{ mW/cm}^2$ and burning times from $t_\text{b} = 50$ to $1000 \text{ s}$ were used. In order to burn shallow holes of relative depth $D \approx 3\%-5\%$ at $4.2 \text{ K}$, burning-fluence densities $P_{\text{b}}/A$ ranged from $10-30 \text{ J/cm}^2$ were needed for the \textit{Rb. sphaeroides} samples, and $P_{\text{b}}/A \approx 25-50 \text{ J/cm}^2$ for \textit{Rps. acidophila}. For $\approx 10\%$ deep holes much higher burning-fluence densities were necessary, $P_{\text{b}}/A \approx 300 \text{ J/cm}^2$ for \textit{Rb. sphaeroides} and $\approx 500 \text{ J/cm}^2$ for \textit{Rps. acidophila}. We noticed that, for a given burning-fluence density and holewidth, the hole depth is wavelength dependent, e.g. holes burnt at 803 nm are about three times deeper than those burnt at 798 nm. A similar effect had previously been observed by us for the isolated LH2 complex [15] and has now been studied in more detail [25]. The holes were probed by fluorescence excitation spectroscopy: the laser, attenuated by a factor of $\approx 500-1000$, was scanned over the spectral region of the hole, and the fluorescence signal was detected in a similar way as described above. Typical scan lengths of approximately 2000 GHz with steps of 5 GHz took $\approx 10-15$ min.

In order to obtain the homogeneous linewidth $\Gamma_{\text{hom}}$, the holewidth $\Gamma_{\text{hole}}$ was plotted as a function of burning-fluence density, $P_{\text{b}}/A$, and extrapolated to $P_{\text{b}}/A \rightarrow 0$. $\Gamma_{\text{hom}}$ was evaluated from the relation $\Gamma_{\text{hole}} \approx 2\Gamma_{\text{hom}} + \sqrt{2\Gamma_{\text{laser}}}$, where $\Gamma_{\text{laser}} \approx 6$ GHz is the bandwidth of the Ti: sapphire laser. We have assumed that the homogeneous line has a Lorentzian shape, and the frequency profile of the laser is Gaussian. The homogeneous linewidth (see section 3.2) is much larger than the laser bandwidth. The samples were immersed in a $^4$He-bath cryostat in which the temperature could be reduced from 4.2 to 1.2 K by lowering the vapour pressure. The temperature was determined from this pressure and also by a calibrated carbon resistor (accuracy $\pm 0.01 \text{ K}$).

3. Results and discussion

3.1. Absorption spectra

In fig. 1 absorption spectra at 4.2 K of membrane preparations from various strains of \textit{Rb. sphaeroides} (figs. 1a, 1b, 1d-1f) and of the isolated B800-820 complex of \textit{Rps. acidophila} (fig. 1c) are given between 760 and 900 nm. The spectral positions of the B800 and B850 bands, their relative distance in cm$^{-1}$, the inhomogeneous linewidths of the B800 bands and the optical densities at 800 nm are reproduced in table 1. Fig. 1a shows the spectrum of the wild-type membrane WT (YY) which, in addition to the LH2 complex, also has the LH1 complex (B875) absorbing at 885 nm. The spectrum of the wild-type LH2-only mutant, B800-850 (YY, "control"), in which the genes of LH1 and RC are absent, is given in fig. 1b. The positions and widths of both B800 and B850 bands are very similar to those of the WT (YY) membrane and of the isolated \textit{Rb. sphaeroides} LH2 complex [15]. The spectrum of the isolated LH2 complex of \textit{Rps. acidophila} B800-820 (FL) (fig. 1c) shows two bands at 800 nm and $\approx 824$ nm. The latter is strongly blue-shifted with respect to the B850 band of \textit{Rb. sphaeroides}. In B800-841 (FY) and B800-839 (YF) (figs. 1d and 1e) the B850 band is blue-shifted by $\approx 10$ nm. In B800-830 (FL) (fig. 1f) the B850 band is blue-shifted by an additional 10 nm. The spectrum of the latter is similar to that of the B800-820 (FL) complex of \textit{Rps. acidophila}, but its B830 band lies at $\approx 829$ nm and is about 40% broader than that of the B820 band (compare figs. 1c and 1f).

We further notice in fig. 1b that the ratio of the intensities of the 800 to the 850 nm band in B800-850 (YY, "control") is smaller than that for the other mutants, which we attribute to repeated irradiation with light and to freeze-thaw cycles [5,26-29]. In fact, in fresh samples of the membranes of the
isolated B800–850 complex the intensity of the B800 band is almost equal to that of the B850 band, which indicates that the isolation procedure did not influence the absorption characteristics of the complex. After using the isolated complex for hole-burning experiments at ≈ 800 nm, and after “mistreating” it by
Table 1
Wavelengths of the maxima of the B800, the (blue-shifted) B850 and the B820 absorption bands of the LH2 complexes of \textit{Rb. sphaeroides} and \textit{Rps. acidophila} studied at 4.2 K; frequency difference between these maxima, \(\Delta\nu\) (in cm\(^{-1}\)); inhomogeneous linewidths \(\Gamma_{\text{inh}}\) of the B800 band (in cm\(^{-1}\)); and optical density at 800 nm, OD\(_{800}\) of 3.5 mm thick samples

<table>
<thead>
<tr>
<th></th>
<th>(\lambda,\text{B800 (nm)})</th>
<th>(\lambda,\text{B850 (nm)})</th>
<th>(\Delta\nu,\text{(cm}^{-1})</th>
<th>(\Gamma_{\text{inh}},\text{(cm}^{-1})</th>
<th>(\text{OD}_{800})</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Rb. sphaeroides}</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>“fresh” isolated LH2 complex</td>
<td>798.4</td>
<td>852.5</td>
<td>795</td>
<td>146</td>
<td>1.07</td>
</tr>
<tr>
<td>“mistreated” isolated LH2 complex</td>
<td>799.1</td>
<td>859.2</td>
<td>875</td>
<td>290</td>
<td>0.23</td>
</tr>
<tr>
<td>WT (YY)</td>
<td>799.2</td>
<td>853.4</td>
<td>795</td>
<td>150</td>
<td>0.37</td>
</tr>
<tr>
<td>B800–850 (YY, “control”)</td>
<td>799.2</td>
<td>855.1</td>
<td>818</td>
<td>182</td>
<td>0.48</td>
</tr>
<tr>
<td>B800–841 (FY)</td>
<td>799.2</td>
<td>842.5</td>
<td>643</td>
<td>165</td>
<td>0.32</td>
</tr>
<tr>
<td>B800–839 (YF)</td>
<td>798.4</td>
<td>839.0</td>
<td>606</td>
<td>145</td>
<td>0.69</td>
</tr>
<tr>
<td>B800–830 (FL)</td>
<td>798.5</td>
<td>828.6</td>
<td>455</td>
<td>180</td>
<td>0.28</td>
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<tr>
<td>\textit{Rps. acidophila} (strain 7050)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>B800–820 (FL)</td>
<td>796.8</td>
<td>824.2</td>
<td>417</td>
<td>186</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Cooling it down and warming it up from 4.2 K to room temperature several times, we noticed that the B800 band not only had lost intensity, but it became asymmetric and almost twice as broad. This effect has been studied in more detail elsewhere [30]. An interesting question in this context is whether the decrease and broadening of the B800 band, which in the literature was attributed to “free” BChl \(a\) molecules absorbing at 775 nm \([27,29]\), affects the energy transfer rate from B800 to B850. We have verified that this is not the case because the “mistreated” isolated LH2 complex, the fresh isolated complex, the WT (YY) membranes (fig. 1a), and the mutant B800–850 (YY, “control”) (fig. 1b) yielded equal holewidths and, therefore, equal energy transfer times, to within \(\pm 4\%\).

3.2. Energy transfer times

Holes burnt into the B800 bands of the various membrane preparations of \textit{Rb. sphaeroides} and the B800–820 complex of \textit{Rps. acidophila} at 4.2 K are shown in fig. 2. We have observed that the holewidths are independent of wavelength between 798 and 805 nm, which indicates that the B800→B800 energy transfer does not take place or, at least, is negligible within the long wavelength part of the B800 band. This was confirmed by us by fluorescence line narrowing (FLN) experiments performed in the same wavelength region \([25]\) (see discussion below). For \(\lambda<798\) nm the holewidths become larger towards the blue side of the B800 band indicating that the energy transfer rate increases and, in particular, that the B800→B800 transfer plays a role. The results are consistent with the B800 fluorescence signal being broad in this region and independent of excitation wavelength \([25]\). In a previous experiment \([15]\) this variation of the holewidth with wavelength at \(\lambda<798\) nm had escaped detection because of the large bandwidth of the laser system used. A more detailed study of the wavelength dependence of the holewidths will be presented elsewhere \([25]\).

The holes burnt between 798 and 805 nm into the B800 band of the mutants other than B800–850 (YY) and into \textit{Rps. acidophila} are all a little broader than the hole in WT (YY) and the isolated LH2 complex: \(\approx 146\) to \(\approx 185\) GHz versus \(\approx 127\) GHz (see fig. 2). Thus, the B800→blue-shifted B850 energy transfer times are a little faster. But before we discuss these differences, we will comment on the HB mechanism.

We think that HB occurs here as a consequence of a change in interaction, after excitation, between the BChl molecules absorbing at 800 nm and the protein to which they are bound \([15]\). One possibility is the BChl \(a\) becomes released from the protein, whereby “free” BChl \(a\) absorbing at \(\approx 775\) nm is formed \([27,29]\). Another possibility, which we favour, is that on excitation a slight relative reorientation of the central magnesium of BChl \(a\) and the axial ligand attached to it occurs, in a similar way as previously proposed for magnesium porphin (MgP) in an \(n\)-oc-
Fig. 2. Holes burnt in the B800 band of the wild-type membrane and of membranes containing four mutated LH2 complexes of *Rb. sphaeroides*, and of the B800-820 complex of *Rps. acidophila*, at 4.2 K. The dots represent data points, the solid lines are Lorentzian fits to the data.

tane crystal [31] where the photoproduct appeared at $\approx 100 \text{ cm}^{-1}$ from the burnt hole, and for BChl a in glasses [32]. In fact, the hole-burning efficiencies in all these systems seem to be similar. For example, for BChl a in triethylamine at 4.2 K, which has a fluorescence lifetime of $\approx 4 \text{ ns}$, holes of a few % depth were obtained with widths of $\approx 1 \text{ GHz}$ using $P_{b/\Delta} / A \approx 0.2 \text{ mJ/cm}^2$ [32]. If we assume that HB in LH2 complexes is due to the same mechanism, we can estimate the burning-fluence density necessary to burn a hole of a few % depth. Since the fluorescence lifetime is about 2 ps for B800 and the holewidths are 100 times larger than for BChl a in glasses, we expect $P_{b/\Delta} / A \approx 10^2 \times 10^3 \times 0.2 \text{ mJ/cm}^2 \approx 20 \text{ J/cm}^2$, which is about the value we needed in the experiments on LH2 complexes. Thus, a HB mechanism for LH2
complexes in which the Mg atom of BChl a is involved is consistent with that proposed for BChl a in glasses [32] and MgP in crystalline n-alkanes [31].

In fig. 3 the holewidths, $\frac{1}{2}\Gamma_{\text{hole}}$, as a function of burning-fluence density, $P_{b}t_{b}/A$, are compared for three samples at 4.2 K. From the extrapolation to $P_{b}t_{b}/A \rightarrow 0$ we obtain the homogeneous linewidths $\Gamma_{\text{hom}}$, summarized in table 2. Holes were also burnt and probed at 1.2 K in B800–841 (FY) and B800–820 (FL) to verify that there is no temperature dependence [15]. Since within the experimental accuracy the holeshape is Lorentzian for all samples studied, we conclude that there is no dispersive kinetics [18] associated with the energy transfer rate.

In order to deduce the energy transfer times from the values of $\Gamma_{\text{hom}}$ we have used a similar reasoning as in ref. [15], which is further supported by new experimental proofs. The homogeneous linewidth is given by

$$\Gamma_{\text{hom}} = \frac{1}{2\pi T_{1}} + \frac{1}{\pi T_{\text{ET}}^{*}(T)} = \frac{1}{2\pi} \left( \frac{1}{\tau_{h}} + \frac{1}{\tau_{\text{ET}}} \right) + \frac{1}{\pi T_{\text{ET}}^{*}(T)}.$$ (1)

$(T_{1})^{-1}$ is the decay rate of excitation for a given molecule, it includes the fluorescence lifetime $\tau_{f}$ and all energy transfer processes to neighbouring molecules. $(T_{\text{ET}}^{*})^{-1}$ is the pure dephasing rate determined by thermally induced fluctuations of the optical transition frequency due to phonon scattering. Whereas the second term is temperature dependent, the first one is assumed to be $T$ independent, at least at very low temperature. If the pure dephasing contributions to $\Gamma_{\text{hom}}$ in these LH2 complexes were of the same order as that found for BChl a in glasses at 4.2 K, then $(\pi T_{\text{ET}}^{*})^{-1} \approx 1 \text{ GHz} \ll \Gamma_{\text{hom}} \approx 60–90 \text{ GHz}$, which is the value found from hole-burning on the red part of the B800 band. Since we did not observe any temperature dependence of $\Gamma_{\text{hom}}$ from 1.2 K up to 30 K [15], we conclude that the second term in eq. (1) can be neglected. The fluorescence decay, $\tau_{f} \approx 1 \text{ ns}$, can also be neglected because its contribution to $\Gamma_{\text{hom}}$ is $(2\pi\tau_{f})^{-1} \approx 0.2 \text{ GHz}$. Moreover, we have observed FLN, i.e. the B800 fluorescence, when excited with a laser in the same red region of the band, is narrow and shifts with excitation wavelength [25]. In addition, the holewidths are independent of wavelength between 798 and 805 nm. From these results we infer that in this wavelength range

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Gamma_{\text{hom}}$ (GHz)</th>
<th>$\tau_{\text{ET}}$ (ps)</th>
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<tbody>
<tr>
<td>WT (YY)</td>
<td>65±4</td>
<td>2.45±0.15</td>
</tr>
<tr>
<td>B800–850 (YY, “control”)</td>
<td>62±2</td>
<td>2.55±0.10</td>
</tr>
<tr>
<td>B800–841 (FY)</td>
<td>94±6</td>
<td>1.70±0.10</td>
</tr>
<tr>
<td>B800–839 (YF)</td>
<td>71±3</td>
<td>2.25±0.10</td>
</tr>
<tr>
<td>B800 830 (FL)</td>
<td>89±4</td>
<td>1.80±0.10</td>
</tr>
<tr>
<td>B800–820 (FL)</td>
<td>79±3</td>
<td>2.00±0.10</td>
</tr>
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</table>

Fig. 3. Holewidth, $\frac{1}{2}\Gamma_{\text{hole}}$, as a function of burning-fluence density, $P_{b}t_{b}/A$, at 4.2 K for the B800–830 (FY) and the B800–830 (FL) mutants of Rh. sphaeroides, and the B800–820 (FL) complex of Rps. acidophila. The extrapolated values of $\frac{1}{2}\Gamma_{\text{hole}}$ for $P_{b}t_{b}/A \rightarrow 0$, deconvoluted from the laser bandwidth $\Gamma_{\text{laser}} \approx 6 \text{ GHz}$, were taken as the homogeneous linewidths, $\Gamma_{\text{hom}}$. The latter were found to be independent of excitation wavelength between 798 and 805 nm and their values yield the B800→(blue-shifted) B850 energy transfer times when exciting in this spectral region. (Δ) $\Gamma_{\text{hom}}$ (B800–830) = 89±4 GHz; (○) $\Gamma_{\text{hom}}$ (B800–820) = 79±3 GHz; (●) $\Gamma_{\text{hom}}$ (B800–850) = 62±2 GHz.
interval the B800 \rightarrow B800 energy transfer is negligible compared to the B800 \rightarrow B850 transfer. This is further supported by the fact that we have not observed low-energy satellite holes when exciting in the red part of the B800 band, as discussed in refs. [16,18]. The values of $\Gamma$ between 798 and 805 nm (see table 2) are, therefore, entirely determined by the energy transfer process from B800 to B850 or, respectively, to the blue-shifted B850 bands in the mutants of Rs. sphaeroides, or the B820 band of Rs. acidophila. Thus, the estimate $\tau_{\text{ET}} = \tau_{\text{B800-B850}} = (2\pi / \Gamma_{\text{B800}})^{-1}$ is valid for BChl a molecules excited on the low-energy side of B800. The value $\tau_{\text{ET}} = 2.55 \pm 0.10$ ps for the LH2-only membranes.

B800-850 (YY, “control”) is to within 4% the same as that for WT (YY), which is consistent with the notion that this mutant contains an intact and fully functional LH2 complex [7]. Further, and contrary to our expectations, the energy transfer time does not monotonically decrease with an increase in overlap (or decrease of spectral distance) of the emission band of B800 and the blue-shifted B850 absorption bands. In fact, we found that the shortest transfer time $\tau_{\text{ET}} = 1.70 \pm 0.10$ ps occurs for B800-841 (FY), and not for B800-820 (FL).

It is instructive to estimate values of $\tau_{\text{ET}}$ assuming three models, all based on Förster’s energy transfer mechanism for weak dipole–dipole coupling [8], and compare them to the hole-burning results. According to Förster the expression for the rate constant is

$$\frac{1}{\tau_{\text{ET}}} = \frac{9 \ln(10)}{128 \pi^5} \frac{\kappa^2}{n^4 N_A \tau_D^0 R_{\text{DA}}^6} \times \int_0^\infty f_D(\tilde{\nu}) \epsilon_A(\tilde{\nu}) \tilde{\nu}^{-4} d\tilde{\nu}. \quad (2)$$

In eq. (2) $N_A$ is the Avogadro number, $\tau_D^0$ represents the pure radiative lifetime of the donor, $R_{\text{DA}}$ is the distance between centers of donor and acceptor, and $n$ is the index of refraction of the medium. In the overlap integral $\tilde{\nu}$ is the energy in cm$^{-1}$, $f_D(\tilde{\nu})$ is the normalized fluorescence spectrum of the donor, and $\epsilon_A(\tilde{\nu})$ is the molar absorption spectrum of the acceptor (in cm$^2$/mmol). $\kappa^2$ is an orientation factor related to the interaction energy of two point dipoles and is given by

$$\kappa^2 = (\cos \varphi_{\text{DA}} - 3 \cos \varphi_D \cos \varphi_A)^2. \quad (3)$$

In eq. (3) $\varphi_{\text{DA}}$ is the angle between the transition dipoles of donor and acceptor, and $\varphi_D$ and $\varphi_A$ are the angles between each dipole and the line connecting them. We have taken a value of $\kappa^2 = 1$ based on the model of ref. [5] in which the directions of the $Q_y$ transition dipole moments of the BChl 800 and BChl 850 molecules are assumed to be approximately parallel to the membrane and on top of each other. Thus, $\varphi_{\text{DA}} = 0^\circ$ and $\varphi_A = \varphi_D = 90^\circ$.

For $\tau_D^0$ we have taken 15 ns, an average value of the radiative lifetime of BChl a in various solvents [33], and for $R_{\text{DA}}$ we have assumed 2.1 nm, which is the upper limit taken from the literature [34]. Since eq. (2) was derived in the dipole approximation of two interacting molecules, a correction should be made if the distance between the centers of the donor and the acceptor molecules becomes comparable to the dipole length [35]. For BChl a, the dipole length may be as large as 1.2 nm [36], whereas $R_{\text{DA}} \leq 2.1$ nm [34]. For the index of refraction we took the value for alanine, $n = 1.45$. This amino acid residue appears many times in the α-peptide chain between the BChl 800 and BChl 850 molecules and is one of the few for which the value of $n$ is known [37].

In our first, crude, calculation we have assumed: (a) large homogeneously broadened bands for the B800 emission and the B850 absorption spectra, and (b) energy transfer from the $Q_y$–$O-O$ transition of B800 to that of B850. Although this model is not realistic for the interpretation of the present experiments because the fluorescence bands are in fact narrow when excited with a laser in the spectra region between 798 and 805 nm and they shift with the excitation wavelength [25], it serves to illustrate the order of magnitude of the energy transfer rates to be expected under such conditions. For the calculation we have taken (1) a width of $\approx 100$ cm$^{-1}$ for the fluorescence band of B800 with a maximum at 805 nm (as obtained when exciting at $\lambda \approx 798$ nm), (2) Gaussian lineshapes for fluorescence and absorption bands, and (3) the areas of all blue-shifted B850 absorption bands were normalized to that of the B850 band of the B800–850 complex. We have further used the extinction coefficient of (supposedly monomeric) B850, $\epsilon_A = 184$ mM$^{-1}$ cm$^{-1}$, taken from ref. [27]. Under these conditions, the values of $\tau_{\text{ET}}$ dif-
fer by more than five orders of magnitude for the various samples, from $\tau_{ET} \approx 6 \times 10^{-12}$ s for B800–830 and B800–820 to $\tau_{ET} \approx 10^{-6}$ s for B800–850. There is a strong discrepancy between these calculated values and those obtained experimentally, which varied only between $\approx 1.7 \times 10^{-12}$ and $2.3 \times 10^{-12}$ s, and this first overly simple model can indeed be discarded.

In the second calculation we have assumed a narrow Q, 0–0 B800 fluorescence band that shifts with excitation wavelength as observed for 798 nm to 805 nm. The width of this Lorentzian-shaped band was taken $\approx 2-3$ cm$^{-1}$, corresponding to that of the homogeneous linewidths of 60–90 GHz obtained from our HB experiments on the various mutants. For the Q, 0–0 B850 absorption band we took the width and shape of the total band as in the first model. The calculated values of $\tau_{ET}$ then not only differ by about five to six orders of magnitude between the various LH2 complexes at a given fluorescence wavelength but, in addition, they show a strong excitation wavelength dependence, ranging from about $\tau_{ET} \approx 9 \times 10^{-12}$ s at 805 nm to $40 \times 10^{-12}$ s at 798 nm for B800–830, and from $6 \times 10^{-6}$ s at 805 nm to $\approx 10^{-1}$ s at 798 nm for B800–850. Since experimentally we did not observe any excitation wavelength dependence of $\tau_{ET}$ between 798 and 805 nm, we also have to disregard the second model. The large discrepancy between the experimental values of $\tau_{ET}$ and the calculated ones in these two models strongly suggests that energy transfer does not occur between the Q, 0–0 band of B800 and that of B850.

In order to explain the rather similar transfer times measured and their independence of wavelength, we have made a third calculation assuming that the energy transfer results from coupling of the narrow Q, 0–0 transition of B800 with a broad vibronic transition of B850 (or of the blue-shifted B850) which lies at $\approx 800$ nm. Such a model is based on the fact that B850 is largely homogeneously broadened at low temperature ($T_{hom} = 220$ cm$^{-1}$ for B850 in the B800–850 complex), probably due to rapid inter-exciton level scattering [16]. It is furthermore known that BChl a in glasses has many vibronic and vibrational bands between 150 cm$^{-1}$ and 1600 cm$^{-1}$ [32,38]. The vibronic frequencies of B850 between 280 cm$^{-1}$ and 920 cm$^{-1}$, which are very similar to those of BChl a, were obtained from satellite holes that appeared in B800 as a result of hole-burning in B850 [16]. It was suggested that the modes in the 700–900 cm$^{-1}$ region are those participating in Förster’s energy transfer [16]. For our calculation we have assumed that the vibronic bands of (blue-shifted) B850 molecules are also broad between 798 and 805 nm and, thus, have a constant absorption $\epsilon_{A,800}$ in this spectral region. We may therefore replace $\vec{p}$ by $\vec{p}_{800}$ in eq. (2), $\epsilon_{\lambda}(\vec{p})$ by $\epsilon_{\lambda,800} = \epsilon_{A,800}$ over a few nm, and $\int_{\vec{p}}^{} f_{\lambda}(\vec{p}) \epsilon_{\lambda}(\vec{p}) \, d\vec{p} = 1$.

With the transfer times $\tau_{ET}$ obtained from the hole-burning experiments and using the same values for $\kappa^2$, $n$, and $\tau_0^3$ as in the other two models, we can calculate the distance $R_{DA}$ for the various LH2 complexes knowing the relative intensity $\epsilon_{A,800}/\epsilon_{A,850}$. We have taken a ratio $\epsilon_{A,800}/\epsilon_{A,850} \approx 0.1$, which was obtained independently from two types of experiments: on the one hand, from the relative intensity of a hole burnt in the B850 band and its satellite hole at 750 cm$^{-1}$ [16]; on the other hand, from the absorption spectrum of a LH2 mutant without B800 band in which the histidine thought to ligate BChl 800 was replaced by serine [39]. If we assume that $\epsilon_{A,800}/\epsilon_{A,850} \approx 0.1$ for all the LH2 complexes, then $R_{DA}$ will vary from 1.7 to 1.5 nm. If, in addition to the coupling of the B800 0–0 transition to a vibronic transition of B850, and equally strong coupling between a vibrational transition of B800 and the 0–0 transition of B850 occurred, the $R_{DA}$ distances would increase by a factor of only $\sqrt[2]{1.12}$; thus, $1.7 < R_{DA} < 1.9$ nm. These values have to be compared to that in the literature for the B800–850 complex of Rh. sphaeroides, in which 2.1 nm had been calculated as an upper limit [34]. Since the membrane has a thickness of $\approx 3.5$ nm [3,4,6], the values of $R_{DA}$ obtained here still look reasonable. A critical point is whether Förster’s eq. (2), which describes the interaction between point dipoles [8], is still valid under the present conditions because the $R_{DA}$ distances are very close to the dipole length of BChl a of $\approx 1.2$ nm [35,36]. Also the refractive index $n$ conceivably might vary between 1.3 and 1.6 [37], the lifetime $\tau_0^D$ between 12 and 18 ns [33], and the extinction coefficient of B850 may become $\epsilon_{A,850} = 368$ mM$^{-1}$ cm$^{-1}$ if the dimer character of B850 is taken into account [27]. The largest uncertainty, however, is given by the orientation factor $\kappa^2$ which can have values from 0 to 4, and which in randomly oriented solutions is equal to $\frac{1}{3}$ [8]. The results show

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that the third model is the only one of the three which gives a transfer time of the correct order of magnitude for the series of mutants studied. Thus, the energy transfer most probably involves a broad vibronic level of the acceptor and/or a vibrational mode of the donor.

4. Conclusions

We have shown that energy transfer times from B800 to (blue-shifted) B850 for four mutated LH2 complexes of Rb. sphaeroides and for the B800–820 complex of Rps. acidophila at 4.2 K vary at most by a factor of 1.5, between 2.5 and 1.7 ps, when excited with a laser between 798 and 805 nm. From the results we conclude that hole-burning is a sensitive technique to detect small differences of a few hundred femtoseconds between energy transfer times of different light-harvesting complexes (see table 2).

Three estimates of the transfer times, all based on Förster’s mechanism [8], have been made. Only one of them is consistent with the hole-burning and the fluorescence line narrowing results obtained. In this model we have assumed that transfer from donor to acceptor occurs through coupling of the narrow Qy 0–0 transition of B800 with a broad vibronic transition of B850, which has approximately constant intensity in the region between 798 and 805 nm. We have also considered that, in addition, transfer may occur involving coupling of a vibrational transition of B800 in resonance with the Qυ 0–0 band of B850. For the calculation we have further assumed that the relative orientations of the BChl a pigments are like those proposed in ref. [5] and are the same for the different mutants, which is justified by circular dichroism experiments [7]. Under these conditions, and from the experimental evidence that the relative intensity of the vibronic band of B850 with respect to that of the Qυ 0–0 band of B850 cannot be larger than −0.1 [16,39], we have estimated the distances between BChl 800 and (blue-shifted) BChl 850 molecules to be $R_{DA} = 1.5$–1.9 nm for the various LH2 complexes studied. These values are somewhat smaller than the upper limit (2.1 nm) reported for B800–850 [34]. In order to judge how realistic $R_{DA}$ and the relative orientations of the BChl a pigments are, we would have to know the three-dimensional structure of LH2 complexes, which so far has not been determined.

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