

## Comparison of the Excited-State Dynamics of Five- and Six-Chlorophyll Photosystem II Reaction Center Complexes

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Received: June 26, 1998; In Final Form: September 2, 1998

Spectral hole-burning experiments have been performed at liquid He temperature on the Q<sub>y</sub>-band of isolated reaction center complexes of photosystem II (PS II RC) containing five (RC-5) and six (RC-6) chlorophyll *a* (Chl *a*) molecules. The aim was to investigate the nature of the redmost shoulder in the absorption spectrum of RC-5 and to identify distributions of “trap” pigments. The “effective” homogeneous line width  $\Gamma'_{\text{hom}}$  was measured at 682 nm as a function of temperature between 1.2 and 4.2 K. It follows a  $T^{1.3 \pm 0.1}$  power law in both complexes and extrapolates to the fluorescence lifetime-limited value,  $\tau_{\text{fl}} = (4 \pm 1)$  ns, for  $T \rightarrow 0$ . These results indicate that the redmost absorbing pigments act as “traps” for the excitation energy. The spectral distribution of these traps was reconstructed from the hole depth measured as a function of excitation wavelength  $\lambda_{\text{exc}}$  and compared to that of RC-6 previously obtained by us (Groot, M. L.; et al. *J. Phys. Chem.* **1996**, *100*, 11488). The maximum of the RC-5 trap distribution lies at  $(682.9 \pm 0.2)$  nm. We discovered a second distribution of fluorescing pigments centered at  $(673.4 \pm 0.5)$  nm in both RC-5 and RC-6. The dependence of  $\Gamma'_{\text{hom}}$  on the delay time  $t_{\text{d}}$  between burning and probing, for the red- and blue-absorbing pigments, is constant for  $t_{\text{d}} \leq 1$  s and increases linearly with  $\log t_{\text{d}}$  for longer delay times. The molecules absorbing at  $\sim 674$  nm, which can be chemically removed, are not free Chl *a* but are bound to a protein with the same mass as that of the RC complex.

### 1. Introduction

The reaction center (RC) of photosystem II (PS II) in green plants is the pigment–protein complex where the primary charge-separation process occurs that eventually leads to the oxidation of water and the evolution of oxygen. The isolated PS II RC, as first prepared from spinach by Nanba and Satoh,<sup>1</sup> consists of the D1 and D2 proteins, the  $\alpha$ - and  $\beta$ -subunits of cytochrome *b559*, and the psbI gene protein. It binds six chlorophyll *a* (Chl *a*) and one or two  $\beta$ -carotenes per two pheophytin *a* (Pheo *a*) molecules.<sup>2</sup> The plastoquinone is lost during isolation. It is assumed that the RC has a “core” comprising four of the six Chl *a* molecules and the two pheophytins and that two Chl *a* molecules are located at the periphery.

Recently, a PS II reaction center containing five Chl *a* per two Pheo *a* molecules was isolated using immobilized metal affinity chromatography.<sup>3</sup> It was concluded from time-resolved absorption-difference spectroscopy that a peripheral Chl *a* absorbing at about 670 nm had been removed.<sup>3</sup> The absorption spectrum at 4.2 K of this RC-5 preparation is characterized by a shoulder at  $\sim 684$  nm.<sup>4</sup> This band is not well resolved in the 4.2 K absorption spectrum of the RC containing six chlorophyll

*a* molecules (RC-6), but it is revealed in the second derivative of the spectrum.<sup>5,6</sup>

The nature of the 684 nm shoulder is still a matter of debate, and several possibilities have been considered. From hole-burning spectroscopy on RC-6, it was suggested that it may be due to “linker” chlorophyll molecules serving to shuttle energy from the nearest CP47 antenna complex to the RC.<sup>7,8</sup> Other groups proposed that the 684 nm shoulder represents the low-energy exciton band of the RC primary electron donor P680<sup>5</sup> or that the 680 and 684 nm features are the result of “heterogeneity” of the absorption band of the primary electron donor.<sup>9,10</sup> It was also attributed to a red-absorbing accessory pigment acting as a “trap” for the excitation energy at low temperature.<sup>11,12</sup> From low-temperature fluorescence and triplet-minus-singlet (T–S) absorption-difference spectroscopy on RC-5, it has recently been proposed that this shoulder originates, partly, from the PS II RC primary donor and, partly, from red-absorbing “traps” in the RC.<sup>4</sup> Since RC-5 has a much more pronounced shoulder at  $\sim 684$  nm than RC-6, it is a well-suited system to obtain more information on the nature of these red-absorbing pigments.

Hole-burning (HB) has proven to be a sensitive method to determine spectral distributions of pigments characterized by their decay time.<sup>12,13</sup> More in particular, it revealed the existence of “trap”-pigments with a fluorescence lifetime of  $\sim 4$  ns and an absorption maximum at  $\sim 682$  nm in the red wing of the Q<sub>y</sub>-absorption band of RC-6 at low temperature.<sup>12</sup> HB, therefore,

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appears an attractive tool to further investigate the nature of the 684 nm shoulder in RC-5 and to verify whether trap pigments indeed exist in such a preparation.

We have carried out persistent and time-resolved HB experiments (from  $10^{-6}$  to  $10^4$  s) between 1.2 and 4.2 K over the entire  $Q_y$ -band of RC-5. The “effective” homogeneous line width  $\Gamma_{\text{hom}}'$ , measured as a function of temperature in the red wing of the band, indeed extrapolates to the fluorescence lifetime-limited value, with  $\tau_{\text{fl}} = (4 \pm 1)$  ns for  $T \rightarrow 0$ , which confirms the presence of traps in this spectral region. From the excitation-wavelength dependence of the hole depth we have also determined the spectral distribution of these trap pigments and compared it to that of RC-6. To our surprise, we have found, in addition to the red-absorbing traps, a second distribution of fluorescing pigments that absorb further to the blue at around 674 nm and have not previously been reported. To investigate the nature of these pigments, we have studied the increase of  $\Gamma_{\text{hom}}'$  with the delay time  $t_d$  between burning and probing caused by spectral diffusion (SD). Recent results obtained in our group for bacterial<sup>14</sup> and plant photosynthetic protein complexes<sup>15</sup> have shown that SD at low temperature differs strikingly for a chromophore bound to a protein as compared with that in a doped organic glass.<sup>16</sup> In this paper we show that the red- and blue-lying fluorescing pigments have a SD behavior very similar to that of the photosynthetic complexes studied in refs 14 and 15. This strongly indicates that the pigments absorbing at  $\sim 674$  nm are bound to the protein. Further purification of the RC-5 samples leads to their disappearance, from which we conclude that this distribution can be assigned to chlorophyll molecules bound to a protein that has the same mass as the PS II RC.

The results presented in this paper demonstrate the high sensitivity of spectral hole burning to unravel the nature and spectral properties of pigments present in very small amounts in samples of photosynthetic pigment–protein complexes.

## 2. Experimental Section

**2.1. Sample Preparation.** The reaction centers of PS II nominally containing six chlorophyll *a* molecules, and here referred to as RC-6, were isolated from CP47 RC complexes of spinach by means of a short Triton X-100 treatment as described in ref 2. The pigment content was determined by means of HPLC pigment analysis (for details, see refs 2 and 4). These PS II RC complexes contained 6.4 Chl *a* and 1.6  $\beta$ -carotenes ( $\beta$ -Car) per 2 pheophytin *a* (Pheo *a*) molecules. For the low-temperature measurements, the samples were diluted in a buffer containing 20 mM BisTris (pH 6.5), 20 M NaCl, 0.03% (w/v) *n*-dodecyl  $\beta$ -D-maltoside (DM), and  $\sim 70\%$  (v/v) glycerol and stored at 77 K when not used.

Photosystem II reaction centers containing approximately five Chl *a* and one  $\beta$ -Car per two Pheo *a* molecules, and which we shall refer to as “original” RC-5, were isolated from pea (*Pisum sativum*) through immobilized Cu-affinity chromatography as described by Vacha et al.<sup>3</sup> with the following slight modification: during all steps of the preparation the concentration of DM was 0.4 mM (0.02% w/v), in contrast to 1–2 mM previously used.<sup>3</sup> The pigment content was determined by HPLC analysis and absorption spectroscopy at 77 K. The loss of one  $\beta$ -Car is probably the cause of the increased instability of RC-5,<sup>3</sup> as compared to that of RC-6. The samples were stored in 50 mM MES, 0.02% DM, and 5 mM imidazole (pH 6.5) at 77 K before studying them. For low-temperature measurements, the samples were diluted in the same buffer with 60% (v/v) glycerol to an optical density OD  $\approx 0.1$ – $0.3$ .

To check the nature of the 674 nm distribution of fluorescing pigments (see below, sections 3.3 and 3.4) in the original RC-5, we submitted these samples to further purification. Two procedures were used. In the first one, original RC-5 was resolubilized with DM and then passed through an ion-exchange column; in the second one, original RC-5 was directly passed through the ion-exchange column. The resolubilization with DM was done at a Chl *a*-concentration of 40  $\mu\text{g}/\text{mL}$  in 5 mM DM, 50 mM MES (pH 6.5) buffer for 10 min at 4 °C. The chromatography was carried out on a Q-Sepharose (Pharmacia) column as follows: the reaction centers were loaded on the column, then washed with 30 mL of a buffer consisting of 50 mM MES and 0.02% DM (pH 6.5), and finally eluted with 80 mM  $\text{MgSO}_4$  in the same buffer. The pigment composition was checked by HPLC and corroborated to be five Chl *a* per two Pheo *a* molecules. The samples were stored in the same buffer as the original RC-5, at 77 K. For low-temperature measurements, both types of “purified” RC-5 samples were subsequently diluted with 60% (v/v) glycerol. Finally, a “degraded” RC-5 sample<sup>3</sup> was prepared in the following way: the original RC-5 preparation was passed through the ion-exchange column and left at room temperature in the dark for 24 h. After “degradation”, and before the sample was studied at liquid He temperature, glycerol was added to a concentration of 60%.

To obtain glasses of good optical quality at liquid He temperature, all samples (RC-5 and RC-6) were slowly cooled (in about 10 min) from room temperature to 77 K by keeping the cuvette (thickness 3 mm) in an empty  $^4\text{He}$ -bath-cryostat of which the outer mantle was filled with liquid nitrogen. Cooling from 77 to 4.2 K was achieved in a few minutes by filling the cryostat with liquid helium. The temperature of the sample, varied between 4.2 and 1.2 K, was controlled by the vapor pressure of  $^4\text{He}$  and measured with a calibrated carbon resistor in contact with the sample. The accuracy in the temperature determination was better than 0.01 K.<sup>12</sup>

**2.2 Apparatus for Optical Spectroscopy and Time-Resolved Hole Burning.** Absorption and fluorescence excitation spectra at liquid helium temperature were taken with a tunable cw dye laser (Coherent 599-21, DCM dye, bandwidth  $\sim 30$  GHz =  $1$   $\text{cm}^{-1}$  without intracavity assembly, amplitude stabilized to  $<0.5\%$ ) pumped by an  $\text{Ar}^+$  laser (Coherent, Innova 310), which was used for excitation with a power density of 2–5  $\mu\text{W}/\text{cm}^2$ . Transmission and fluorescence signals were measured simultaneously at  $90^\circ$  with respect to each other with two cooled photomultipliers (PM, EMI 9658R). Details of how these spectra were obtained can be found in ref 12.

Fluorescence spectra were recorded by exciting the sample at  $\lambda_{\text{exc}} = 620$  nm with the cw dye laser, but with a power density of 5–10  $\text{mW}/\text{cm}^2$ , and monitoring the signal through a scanning 0.85 m double monochromator (Spex 1402, resolution  $\sim 5$   $\text{cm}^{-1}$ ) with the same type of cooled photomultiplier.

Time-resolved hole-burning (HB) experiments were carried out in a setup as described in refs 15 and 17, but with different lasers (see below). We used a sequence of three laser pulses: (i) in order to obtain a baseline, a probe pulse at low intensity was applied during which the frequency of the laser was scanned over the spectral region of interest; (ii) to create a hole, a burn pulse at higher intensity was applied during which the frequency of the laser was fixed; (iii) in order to monitor the hole after a variable delay time  $t_d$ , a second probe pulse at low intensity was applied during which the frequency of the laser was scanned again over the same spectral region as before burning.

For the hole-burning experiments, two types of lasers were used, depending on the time scale of the experiments. For delay

times shorter than 100 ms, we used current- and temperature-controlled single-mode diode lasers (Toshiba TOLD 9140,  $P_{\max} = 20$  mW,  $680 < \lambda < 687$  nm, and a TOLD 9225,  $P_{\max} = 10$  mW,  $664 < \lambda < 675$  nm, both with bandwidth  $\Gamma_{\text{laser}} \approx 3$  MHz). For delay times longer than  $\sim 100$  ms, the same cw dye laser as mentioned above was used, but in its single-frequency version (with an intracavity assembly, laser bandwidth  $\Gamma_{\text{laser}} \approx 2$  MHz). The wavelength of the lasers was calibrated with a Michelson interferometer (home-built, resolution  $\sim 50$  MHz), and its mode structure was monitored with a confocal Fabry–Perot Etalon (Tropel 240-2, FSR = 1.5 GHz). The area  $A$  of the laser beam on the sample varied from  $A = 0.02$  to  $0.5$  cm<sup>2</sup>, depending on HB efficiency and absorption intensity of the pigments involved.

Burning-power densities  $P/A = 1$   $\mu\text{W}/\text{cm}^2$  to  $20$  mW/cm<sup>2</sup> were used for burning times between  $10$   $\mu\text{s}$  and  $\sim 100$  s. Thus, burning-fluence densities varied from  $Pt_b/A = 0.2$   $\mu\text{J}/\text{cm}^2$  to  $10$  mJ/cm<sup>2</sup>. Holes were probed in fluorescence excitation with the same laser, but its power was attenuated by a factor varying from  $10$  to  $10^3$ , depending on (i) whether the holes are transient or persistent and (ii) the duration of the probe pulse.

The delay time  $t_d$  between burning and probing the hole was varied between  $10$   $\mu\text{s}$  and  $5$  h. For delay times shorter than  $30$  s, the burn and probe pulses were produced with two acoustooptic modulators (AOMs, Isomet 1206C, center frequency =  $110$  MHz) in series. Two AOMs were used, instead of one, to reduce the intensity of the laser light leaking through when the gating pulses were switched off (suppression better than  $10^6$ ). For delay times longer than  $30$  s, no AOMs were used; the intensity of the probe pulse was reduced by using an optical density filter.

The fluorescence signal of the hole was detected with the PM. To separate the fluorescence signal from the scattered laser light, a few long-wavelength-pass filters were used, in most cases Schott RG 715 (total thickness  $\sim 1.2$  cm) such that  $\lambda_{\text{det}} \geq 715$  nm. For delay times shorter than  $30$  s, the signal from the PM was amplified with a load resistor and a differential preamplifier (HMS-Elektronik, model 568) or a band-pass amplifier (Ithaco, model 1201). To increase the signal-to-noise ratio, persistent holes with  $2$  ms  $\leq t_d \leq 30$  s were averaged about  $50$ – $100$  times with a digital oscilloscope (LeCroy 9360, bandwidth  $300$  MHz). Transient holes, which fill in with the lifetime of the triplet state ( $\tau_{\text{tr}} \approx 1$ – $2$  ms), were observed at  $t_d \leq 2$  ms and were averaged  $10^3$ – $10^4$  times. For delay times longer than  $30$  s, the signal from the PM was amplified with an electrometer (Keithley, model 610 C), and a hole was measured only once, but averaged point by point  $\sim 1000$  times with a PC.

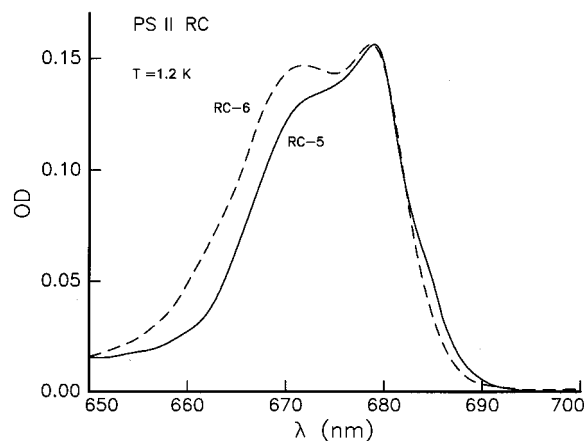
The hole profiles were well fitted with Lorentzian curves. The effective homogeneous line width  $\Gamma'_{\text{hom}}$  was obtained from the value of the hole width extrapolated to zero burning-fluence density  $Pt_b/A \rightarrow 0$ ,  $\Gamma_{\text{hole},0}$ , in the following way. For delay times  $t_d < 30$  s, we took  $t_b \approx t_d$ . In this case

$$\Gamma'_{\text{hom}}(t_d) \approx \frac{1}{2}\Gamma_{\text{hole},0}(t_b, t_b) - \Gamma_{\text{laser}} \quad (1)$$

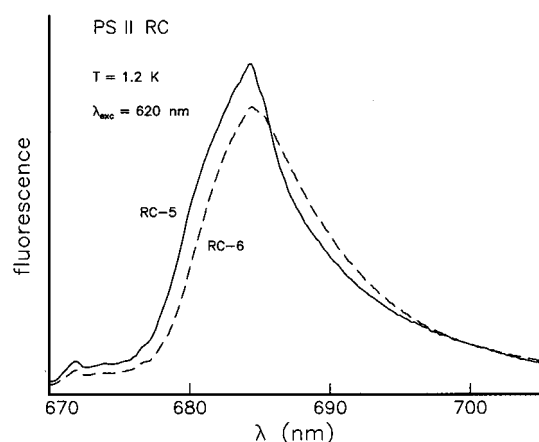
For delay times  $t_d > 30$  s,  $t_b < t_d$  and  $\Gamma'_{\text{hom}}$  was obtained as described in refs 17 and 18

$$\Gamma'_{\text{hom}}(t_d) = \Gamma_{\text{hole},0}(t_b, t_d > t_b) - \frac{1}{2}\Gamma_{\text{hole},0}(t_b, t_b) - \Gamma_{\text{laser}} \quad (2)$$

where  $\Gamma_{\text{hole},0}(t_b, t_b)$  is the hole width extrapolated to zero burning-fluence density for a delay time  $t_d = t_b$ . The value of  $\Gamma_{\text{laser}}$  was taken to be equal to  $\sim 2$  MHz for the dye laser and  $\sim 3$  MHz for the diode laser.



**Figure 1.** Absorption spectra at 1.2 K of the  $Q_y$ -region of the isolated reaction center (RC) complexes of photosystem II (PS II) containing five (RC-5, solid line) and six (RC-6, dashed line) chlorophyll *a* molecules per RC. The original RC-5 sample was prepared as described in ref 3, with a slight modification.

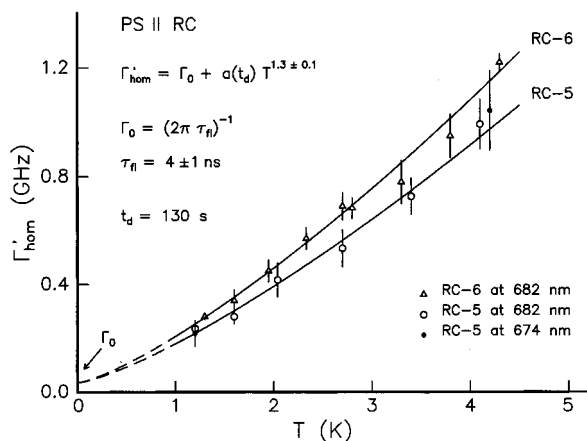


**Figure 2.** Broad-band fluorescence spectra of the RC-5 and RC-6 samples at 1.2 K, excited at 620 nm. The spectra were normalized on their areas.

### 3. Results and Discussion

**3.1. Absorption and Fluorescence Spectra.** The absorption spectra at 1.2 K of the  $S_1 \leftarrow S_0$  0–0 band of the RC-5 and RC-6 PS II RC complexes are shown in Figure 1, normalized at their maximum at  $\sim 680$  nm. The spectra are similar to those at 4.2 K reported in ref 4. RC-5 shows a shoulder in the red wing at  $\sim 684$  nm, which is not directly seen in RC-6. It also has less absorption around 670 nm than RC-6. The shoulder at 684 nm was attributed in ref 4 to absorption by the inner chlorophyll-like molecules forming the core pigments of the reaction center. The decrease of intensity at  $\sim 670$  nm in RC-5 is due to removal of one of the blue-absorbing peripheral chlorophyll molecules from RC-6.<sup>3,4</sup>

Figure 2 shows the fluorescence spectra of RC-5 and RC-6 at 1.2 K, excited at 620 nm. They have been normalized on their areas because their fluorescence yields are about equal.<sup>4,11</sup> The two spectra have their maxima at  $684.4 \pm 0.3$  nm. In accordance with previous results,<sup>4</sup> the spectrum of RC-5 is  $\sim 10\%$  narrower than that of RC-6. It is also more asymmetric. The shape of these spectra is not yet understood. It has been suggested in ref 19 that the asymmetric shape for RC-6 may be due to two Gaussian distributions of fluorescing pigments that absorb at different wavelengths. Since the same number of pigments gives rise to the 684 nm feature in RC-5 as in RC-6, the more asymmetric shape of the fluorescence spectrum of



**Figure 3.** Temperature dependence of the effective homogeneous line width  $\Gamma'_{\text{hom}}$  for RC-5 and RC-6 between 1.2 and 4.2 K. Burning wavelengths:  $\lambda_b = 682$  nm (open symbols) for both samples, and 674 nm for RC-5 (closed circles). The delay time between burning and probing the hole was  $t_d = 130$  s.  $\Gamma'_{\text{hom}}$  follows a  $T^{1.3 \pm 0.1}$ -power law and extrapolates to  $\Gamma_0 = (2\pi\tau_f)^{-1} = (40 \pm 10)$  MHz for  $T \rightarrow 0$  in both RC-5 and RC-6. The value of  $T_1$  corresponds to the fluorescence lifetime  $\tau_f = (4 \pm 1)$  ns of chlorophyll. The two data points at 1.2 and 4.2 K for RC-5 excited at 674 nm fall on the same curve, within the error bars, as the data at 682 nm.

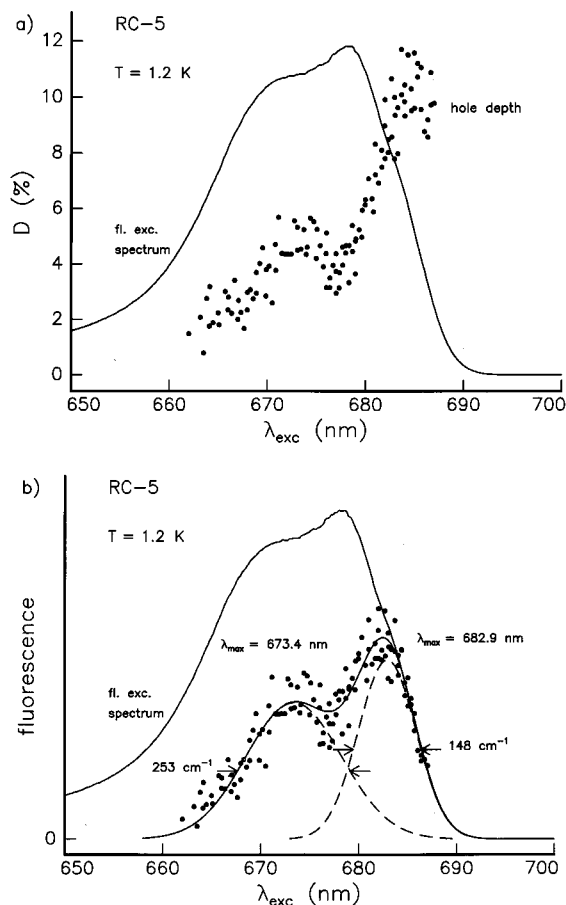
RC-5 may be due to a red-shift of the redmost Gaussian distribution, as proposed in ref 4. The spectra also show broad emission between 670 and 675 nm with a small peak around 672 nm, which corresponds to the  $1242 \text{ cm}^{-1}$  vibration of free Chl *a*.<sup>20</sup> To estimate the amount of free Chl per RC in our samples, we have integrated the fluorescence spectra of RC-5 and RC-6 from 660 nm (not shown) to 676 nm and divided the areas by the total area between 660 and 705 nm. The resulting ratios are 0.057 and 0.044, respectively. Since the fluorescence yield of free Chl *a* ( $\phi_f \sim 0.32^1$ ) is about four times higher than that of the RCs ( $\phi_f \sim 0.08$  for both RC-5 and  $\sim 0.07$  for RC-6<sup>4,11</sup>), we estimate the amount of free Chl per RC in the two samples to be about the same and only  $(1.3 \pm 0.2)\%$ .

**3.2. Identification of Trap Pigments and Their Spectral Distributions.** To prove the presence of trap pigments in the red wing of RC-5 and to compare them to those in RC-6<sup>12</sup> we have first measured the temperature dependence of the effective homogeneous line width  $\Gamma'_{\text{hom}}$  in the red wing of the 0–0 band at 682 nm between 1.2 and 4.2 K, at a delay time  $t_d = 130$  s. Figure 3 shows the results for RC-5 (open circles) as compared to those for RC-6 (open triangles). The data follow a  $T^{1.3 \pm 0.1}$ -power law, as previously found in doped organic glasses at low temperature<sup>17,22,23</sup> and very recently also in other photosynthetic protein complexes:<sup>12,14,16,24</sup>

$$\Gamma'_{\text{hom}} = \Gamma_0 + a(t_d)T^{1.3 \pm 0.1} \quad (3)$$

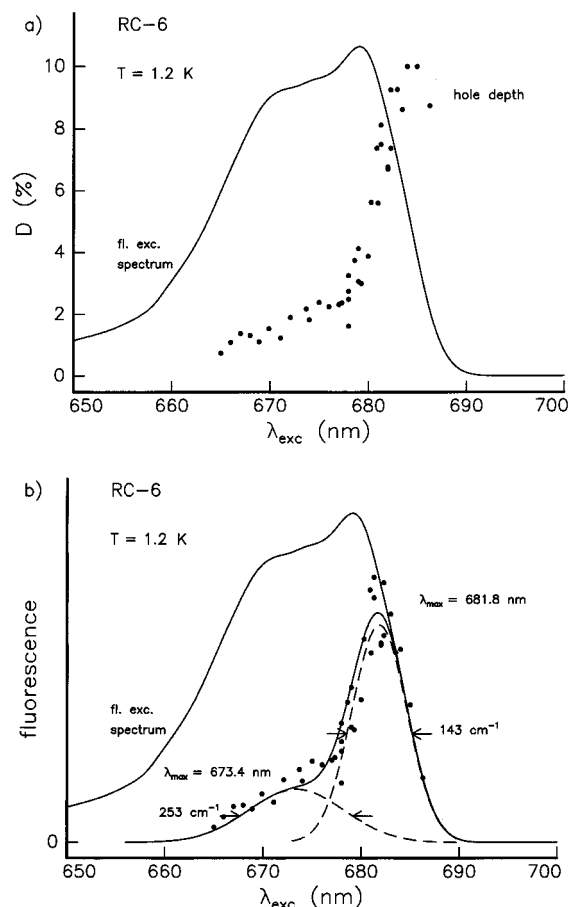
The two curves in Figure 3 extrapolate to a common value  $\Gamma_0 = (2\pi\tau_f)^{-1} = (40 \pm 10)$  MHz for  $T \rightarrow 0$ , with  $\tau_f = (4 \pm 1)$  ns the fluorescence lifetime of Chl *a*.<sup>12,24</sup> The results prove that there are trap pigments in RC-5 absorbing at the redmost wing of the 0–0 band which do not transfer energy and only fluoresce. Such traps for the excitation energy were previously observed by us for RC-6.<sup>12</sup> We associate the pigment(s) responsible for these traps to the “core” chlorophyll-like molecules of the RC, in agreement with the results described in refs 4 and 19.

We have also determined the spectral distributions of the trap pigments in RC-5. They were obtained with the same method as previously used for RC-6,<sup>12</sup> but the depth  $D(\%)$  of the holes



**Figure 4.** (a) Relative hole depth  $D(\%)$  as a function of excitation wavelength  $\lambda_{\text{exc}}$  for RC-5 at 1.2 K together with the fluorescence excitation spectrum in the  $Q_y$ -region. The experiments were carried out with a burning-fluence density  $P_{\text{th}}/A = 0.8$  mJ/cm<sup>2</sup>. (b) Spectral distributions of the fluorescing pigments of the RC-5 (dashed lines) reconstructed within the fluorescence excitation spectrum. They were obtained by multiplying the data of Figure 4a by the fluorescence excitation spectrum at each  $\lambda_{\text{exc}}$ . The resulting curve (solid line) was fitted with two Gaussian curves (dashed lines).

was now measured over a larger spectral region, from  $\lambda_{\text{exc}} = 660$  to 690 nm. The results are shown in Figure 4a,b. Holes were burnt at 1.2 K as a function of excitation wavelength  $\lambda_{\text{exc}}$ , keeping the burning-fluence density constant ( $P_{\text{th}}/A = 0.8$  mJ/cm<sup>2</sup>) (Figure 4a). Multiplying the relative hole depths  $D(\%)$  by the profile of the fluorescence excitation spectrum then yields the trap distribution (Figure 4b). Surprisingly, we have not only found a trap distribution in the red wing of the band at  $\sim 682$  nm but also a shallower distribution centered at  $\sim 674$  nm. To check whether this second distribution is real, we repeated the experiments at various burning-fluence densities. The data have been fitted with two Gaussian distributions. The redmost one has its maximum at  $(682.9 \pm 0.2)$  nm and a width of  $(148 \pm 7)$  cm<sup>-1</sup>. The distribution further to the blue has its maximum at  $(673.4 \pm 0.5)$  nm and a width of  $(253 \pm 20)$  cm<sup>-1</sup>. The heights of the distributions were normalized in such a way that the redmost distribution fits the red wing of the fluorescence excitation spectrum (see Figure 4b). The width of the distribution at 682.9 nm is similar to that of the trap distribution in RC-6<sup>12</sup> but absorbs about 1 nm further to the red. These distributions, which were here obtained within the fluorescence excitation spectrum, differ from those reported in ref 4, which were obtained within the absorption spectrum by deconvolution with five Gaussians. The redmost distributions in ref 4 had maxima at 684.1, 679.4, and 675.2 nm, with widths of  $\sim 83$ ,

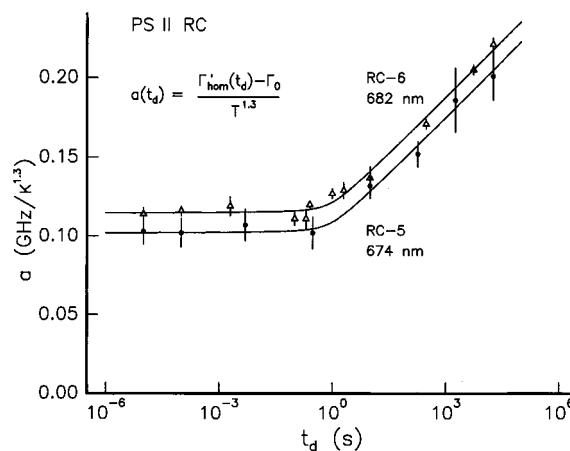


**Figure 5.** (a) Relative hole depth  $D(\%)$  as a function of excitation wavelength  $\lambda_{\text{exc}}$  for RC-6 at 1.2 K together with the fluorescence excitation spectrum. The experiments were performed with  $P_{t_b}/A = 0.8 \text{ mJ/cm}^2$ , as in Figure 4a. (b) Spectral distributions of the RC-6 fluorescing pigments (dashed lines) reconstructed within the fluorescence excitation spectrum (see also Figure 4b).

120, and  $\sim 100 \text{ cm}^{-1}$ , respectively. The deconvolution method,<sup>4</sup> however, does not yield distributions characterized by their decay times, as hole-burning does.<sup>12</sup>

The holes used to obtain the data of Figure 4a at 1.2 K were slightly power broadened ( $\Gamma_{\text{hole}} = 0.7 \text{ GHz}$ ). Their widths were constant from 660 to 686 nm, suggesting that the two spectral distributions at 673.4 and 682.9 nm indeed correspond to fluorescing pigments. To verify this, we have determined the value of  $\Gamma'_{\text{hom}}$  (by extrapolation of  $\Gamma_{\text{hole}}$  to  $P_{t_b}/A \rightarrow 0$ ) at 674 nm, at 1.2 and 4.2 K. The data points, which are plotted in Figure 3 (closed circles), fall on the same curve (within the error bars) as those obtained at 682 nm (open circles); i.e., they extrapolate to  $\Gamma_0$ . The results, therefore, prove that the distribution at  $\sim 674 \text{ nm}$  indeed consists of fluorescing pigments. This is the first time, to our knowledge, that such a “blue” distribution has been identified in a PS II RC sample.

To check whether a 674 nm distribution is also present in RC-6, we have performed the same type of measurements as shown in Figure 4a for this sample. The results, illustrated in Figure 5a,b, show that holes may be burnt around 674 nm in RC-6, but their depths are much smaller than in RC-5. The distributions of Figure 5b, which are qualitatively similar to those of RC-5, have been plotted by assuming (i) that the redmost one has the same position and width as previously found for RC-6 ( $\lambda_{\text{max}} = (681.8 \pm 0.3) \text{ nm}$  and width =  $(143 \pm 5) \text{ cm}^{-1}$ )<sup>12</sup> and (ii) that the blue distribution originates from the same type of pigments as in RC-5 with the same position and



**Figure 6.** Coupling constant  $a(t_d)$  as a function of  $\log t_d$  between  $10^{-6}$  and  $10^5 \text{ s}$ , for RC-6 excited at 682 nm (detected at  $\lambda_{\text{det}} \geq 715 \text{ nm}$ )<sup>15</sup> and RC-5 excited at 674 nm (detected at  $\lambda_{\text{det}} \geq 695 \text{ nm}$ ). The value of  $a(t_d)$  is constant for  $10^{-6} \text{ s} \leq t_d \leq 1 \text{ s}$ . The data in this time span are probably determined by “pure” dephasing. For  $t_d \geq 1 \text{ s}$  and up to 5 h,  $a(t_d) \propto \log t_d$  for both samples. For a discussion, see text.

width as in Figure 4b. The data points are well fitted with these assumptions. It is striking that the relative intensity of the 674 nm distribution with respect to that of the 682 nm distribution is much smaller in RC-6 (Figure 5b) than in RC-5 (Figure 4b). This strongly indicates that the 674 nm distribution cannot be due to free Chl *a*, since the fluorescence spectra in Figure 2 yielded a content of free Chl *a* roughly equal for the two samples. Furthermore, free Chl *a* absorbs at  $\sim 669\text{--}670 \text{ nm}$ <sup>20</sup> and not at  $\sim 674 \text{ nm}$ . The slight difference in the relative intensity of the 682 nm distribution with respect to that of the fluorescence excitation spectrum in RC-5 and RC-6 (compare Figures 4b and 5b) may be due to the small difference in the fluorescence quantum yields of the two samples.<sup>4</sup> To get an idea about the nature of the blue distribution, we have performed the experiments described in the following two sections.

**3.3. Are the 674 nm Pigments Bound to the Protein?** To test if the pigments absorbing around 674 nm are bound to the protein, we have performed spectral diffusion (SD) experiments. We recently found that the SD behavior of photosynthetic pigment–protein complexes is different from that in doped organic glasses.<sup>16</sup> In the latter,  $\Gamma'_{\text{hom}} \propto \log t_d$  for experimental time scales between nanoseconds and hours,<sup>17,23</sup> whereas in the subcore complexes RC-6, CP47 and CP47 RC of PS II,<sup>15</sup> and in the subunits B820 and B777 of the LH1 light-harvesting complex of purple bacteria,<sup>14</sup>  $\Gamma'_{\text{hom}}$  remains constant for short delays ( $t_d \leq 10 \text{ ms} - 1 \text{ s}$ ) and  $\Gamma'_{\text{hom}} \propto \log t_d$  for longer delay times. The results suggest that only very low-frequency modes (with rates  $< 1\text{--}100 \text{ Hz}$ ) are involved in structural relaxation of these photosynthetic pigment–protein complexes at liquid He temperatures.

By measuring  $\Gamma'_{\text{hom}}$  as a function of  $t_d$ , it is, therefore, possible to distinguish whether a pigment is free (like in glasses) or bound to the protein.<sup>14</sup> We have performed this type of experiment on RC-5, burnt at  $\lambda_b = 674 \text{ nm}$ , as a function of delay time  $t_d$  between  $10^{-6}$  and  $10^4 \text{ s}$ , at 1.2 and 4.2 K, and compared the results with those obtained for RC-6 burnt at  $\lambda_b = 682 \text{ nm}$ .<sup>15,16</sup> In Figure 6, the coupling constant  $a(t_d)$ , which is given by

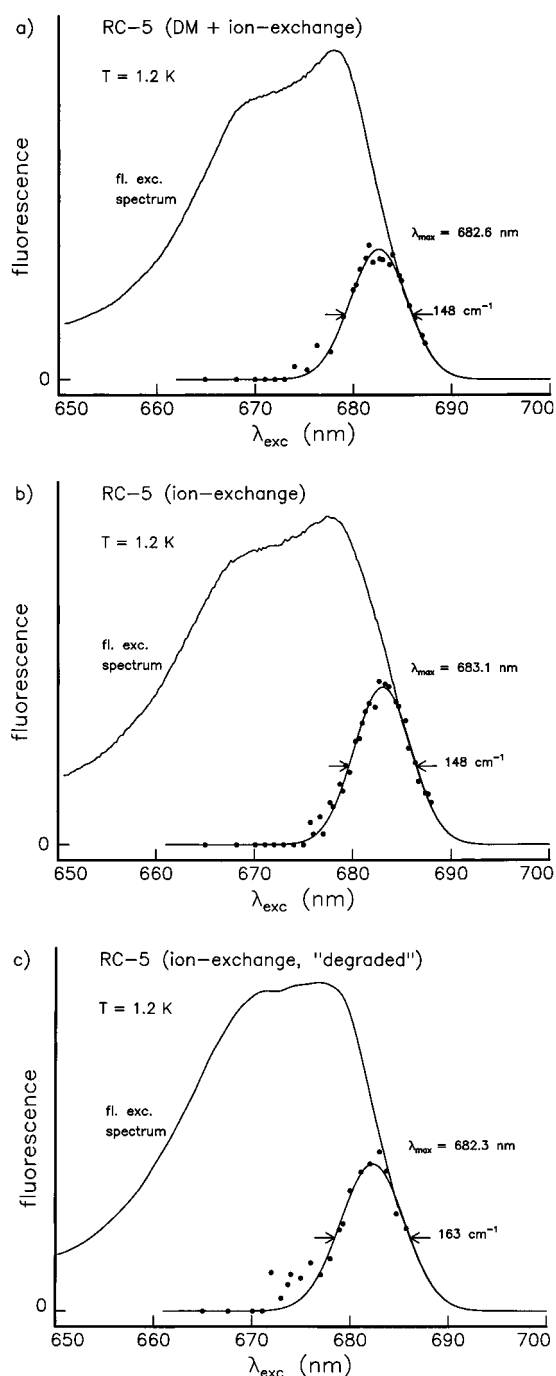
$$a(t_d) = \frac{\Gamma'_{\text{hom}}(t_d) - \Gamma_0}{T^{1.3}} \quad (4)$$

has been plotted as a function of the logarithm of  $t_d$ . RC-5 at

674 nm behaves similarly to RC-6 at 682 nm: for  $t_d \leq 1$  s,  $a(t_d) = \text{constant}$ , whereas for longer delay times,  $a(t_d) \propto \log t_d$ . The onset of SD occurs at about the same delay time ( $t_d \approx 1$  s) for both samples, but the value of  $a(t_d)$  at all delay times is somewhat smaller for RC-5 (see also Figure 3). This may indicate that the RC-5 pigments have a slightly weaker interaction with the protein than RC-6. Notice further that the slope  $da/d \log t_d$ , which is proportional to the amount of SD, is the same for RC-5 and RC-6. Since we have recently found that this slope is correlated with the mass of the protein,<sup>15</sup> we infer from the results in Figure 6 that the protein masses participating in SD are about equal for the two samples. This seems reasonable because they only differ by one Chl *a* pigment ( $\sim 1$  kDa), whereas their protein mass amounts to  $\sim 110$  kDa. We conclude that the Chl *a* molecules giving rise to the 674 nm distribution are bound to a protein of a mass equal to that of the monomeric PS II RC and are, therefore, not the remainder of core antennas left over during isolation of the PS II RC.

**3.4. The Nature of the Pigments at 674 nm.** One could imagine that the pigments absorbing at  $\sim 674$  nm might be either chlorophyll molecules nonstoichiometrically bound to the protein or chlorophylls nonspecifically attached to a protein of the size of the PS II RC. Holzwarth and co-workers<sup>25,26</sup> analyzed the absorption spectra of RC-6 at four temperatures between 10 and 277 K by deconvolving them with many distributions. They included two to three minor ones, which they assigned to nonstoichiometrically bound chlorophylls<sup>25</sup> arising from the fact that the ratio of chlorophyll per two pheophytin molecules is, in general, not an integer number (expected to be six in RC-6).<sup>2</sup> One of these distributions was reported to absorb at  $\sim 675$  nm,<sup>25</sup> a wavelength close to our 674 nm distribution when considering the accuracy of the analysis. Furthermore, the intensity of this Chl distribution as compared to that of the trap Chls at 682 nm was reported to vary between 0.02 and 0.35, depending on sample preparation.<sup>25</sup> Our RC-6 sample showed a ratio of Chl<sub>674</sub>/Chl<sub>682</sub>  $\sim 0.2$  (see Figure 5b), which is within this range. A distribution at  $\sim 675$  nm with comparable relative intensity was also reported in ref 4 but was not assigned. Thus, we would be tempted to associate the 674 nm distributions in Figures 4a,b and 5a,b to chlorophyll molecules nonstoichiometrically bound to the protein, but one would then expect that these pigments transfer energy "downhill", which they do not.

We have checked, in addition, whether the relative intensity of the 674 nm distribution indeed depends on sample preparation, as suggested in ref 25. For this purpose we have reconstructed the distributions of further purified RC-5 preparations at 1.2 K. The results are shown in Figure 7a, b, and c. The experiments were all carried out at the same burning-fluence density ( $P_{t_b}/A = 0.8$  mJ/cm<sup>2</sup>) as used to obtain the results of Figures 4 and 5. Figure 7a shows the spectral distribution for a RC-5 sample that was first resolubilized with 5 mM dodecyl maltoside (DM) and, subsequently, passed through an ion-exchange column. Although the red trap distribution at  $\sim 682$  nm is still present, the 674 nm distribution disappeared. This experiment shows that the protein-bound Chl *a* pigments in the 674 nm distribution are washed away either during DM solubilization and/or ion-exchange chromatography. We further notice in Figure 7a that the fluorescence excitation spectrum does not show the red shoulder at  $\sim 684$  nm (compare to Figure 4). This is probably the reason for the  $\sim 0.3$  nm blue-shift of the red trap distribution, from 682.9 nm in the original RC-5 preparation (Figure 4a) to 682.6 nm in the sample treated with DM + ion-exchange (Figure 7a).



**Figure 7.** (a) Spectral distribution of trap pigments of a RC-5 sample resolubilized with dodecyl maltoside (DM) and, subsequently, passed through an ion-exchange column. (b) The same as (a), but the original RC-5 sample was passed only through an ion-exchange column. (c) The same as (b), but for a "degraded" RC-5 sample (RC-5 was left at room temperature in the dark for 24 h before being measured). The distributions in (a), (b), and (c) are all shown within their fluorescence excitation spectra. The 674 nm distribution was not observed in any of these samples, indicating that the blue distribution consists of protein-bound chlorophyll molecules that can easily be removed from the PS II RC sample but, when present, are not detectable with conventional techniques. For a discussion, see text.

We have also measured a sample of RC-5 which, after isolation (original RC-5), was directly put through the ion-exchange column, without previous DM treatment. The results are shown in Figure 7b. The distribution at 674 nm is not present, as in the sample treated with DM, but the red trap distribution has about the same spectral position and width as the distribution of the original RC-5 (see Figure 4a,b). Also

the fluorescence excitation spectra (compare Figures 4b and 7b) and the absorption spectra at room temperature (not shown) of the two samples are very similar. From these results we conclude that the 674 nm distribution can be easily removed from the sample by passing the original RC-5 preparation through an ion-exchange column. By comparing the results of Figure 7a,b, we further see that if these complexes are subject to high concentrations of DM (Figure 7a), structural changes seem to occur in the RC that are reflected in the blue-shift of the 684 nm absorbing pigments.

In Figure 7c, the effect of degradation is shown for an original RC-5 sample that was first passed through an ion-exchange column and then left for 24 h in the dark at RT (see section 2.1).<sup>3</sup> A significant change in the fluorescence excitation spectrum is observed: the red shoulder at 684 nm has disappeared, and the broad peak at ~670 nm has increased. The red trap distribution is now blue-shifted by 0.6–0.8 nm (from ~683 to ~682.3 nm) and broadened (163 vs 148 cm<sup>-1</sup>). Since the 674 nm distribution did not reappear after “degradation”, we conclude that the protein-bound pigments absorbing at this wavelength are not the result of this process. Thus, they are molecules nonspecifically attached to the periphery of a PS II RC, which is probably inactive as revealed by the absence of “downhill” energy transfer.

#### 4. Conclusions

From the temperature dependence of the “effective” homogeneous line width  $\Gamma'_{\text{hom}}$  at liquid He temperatures, we conclude that the redmost absorbing pigments at ~683 nm in RC-5 act as “traps” for the excitation energy, as previously found for RC-6.<sup>12</sup> They only fluoresce and are not involved in energy transfer. Their interaction with the surrounding protein may be somewhat weaker than for RC-6. Since RC-5 has one Chl *a* molecule less than RC-6, the removed Chl *a* responsible for the absorption at ~670 nm must belong to the periphery of the RC, whereas the red “trap”-pigments must be part of the inner core of chlorophyll-like molecules within the RC. This red trap distribution in RC-5, which was reconstructed from hole depth versus  $\lambda_{\text{exc}}$  experiments, has the same inhomogeneous width as that in RC-6 (~148 cm<sup>-1</sup>) suggesting that the disorder in RC-5 is about the same as in RC-6. Its maximum, however, is red-shifted by ~1 nm (681.8 nm in RC-6 to 682.9 nm in RC-5), which confirms that the interaction of the red-absorbing pigments with the surrounding protein is slightly different in the two samples.

The high sensitivity of the hole-burning technique has allowed us, further, to identify a second distribution of fluorescing pigments at ~674 nm in both the RC-5 and RC-6 samples, not previously reported. Since the relative intensity of this “blue” distribution with respect to the “red” one is much lower in RC-6 than in the “original” RC-5, but the amounts of free Chl per RC are equal for the two samples, it rules out the possibility of attributing the blue distribution to free Chl *a*. Because these 674 nm chlorophylls can be removed from the sample by passing it through an ion-exchange column, and they do not reappear after sample “degradation” (RC-5 left for 24 h in the dark at RT), they are not a product of this degradation. From these results and from the delay-time dependence of  $\Gamma'_{\text{hom}}$ , we conclude that the 674 nm distribution, which is present in small amounts and has not been detected by conventional methods, consists of fluorescing pigments bound to a protein that has the same mass as the PS II RC complex but is probably photochemically inactive.

**Acknowledgment.** We thank H. van Roon for providing us with the RC-6 samples. F.V. acknowledges the European Science Foundation for a travel grant to London, and The Netherlands Foundation for Chemical Research (SON) for a “visitors grant” to Leiden. His biochemical work was supported by the grants GACR P121 and MSMT VS 96085. J.B. acknowledges financial support from the Biotechnology and Biological Science Research Council (BBSRC). We thank J.H. van der Waals for constructive comments on the presentation of the manuscript. The spectroscopic investigations in Leiden were supported by the Netherlands Foundation for Physical Research (FOM) and Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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