A Thermal Broadening Analysis of Absorption Spectra of the D1/D2/Cytochrome *b*-559 Complex in Terms of Gaussian Decomposition Sub-bands

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ABSTRACT: Absorption spectra of the isolated D1/D2/cytochrome *b*-559 complex have been measured in the temperature range 80−300 K. All spectra were analyzed in terms of a linear combination of Gaussian bands and the thermal broadening data interpreted in terms of a model in which the spectrum of each pigment site is broadened by (a) a homogeneous component due to linear electron−phonon coupling to a low-frequency protein vibration and (b) an inhomogeneous component associated with stochastic fluctuations at each pigment site. In order to obtain a numerically adequate description of the absorption spectra, a minimum number of five sub-bands is required. Further refinement of this sub-band description was achieved by taking into account published data from hole burning and absorption difference spectroscopy. In this way, both a six sub-band description and a seven sub-band description were generated. In arriving at the seven sub-band description, the original five sub-band wavelength positions were essentially unchanged. Thermal broadening analysis of the seven sub-band description yielded data which displayed the closest correspondence with the literature observations. The wavelength positions of the sub-bands were near 661, 667, 670, and 675 nm, with two bands near 680 and 684 nm. The two almost isoenergetic sub-bands near 680 nm, identified as P680 and pheophytin, have optical reorganization energies around 40 and 16 cm^{-1}, respectively. All other sub-bands, identified as accessory pigments, have optical reorganization energies close to 16 cm^{-1}. The half-bandwidths at room temperature for accessory pigments and pheophytin are between 10 and 12 nm (210−250 cm^{-1}) with homogeneously broadened line widths in the range 9−11 nm (186−232 cm^{-1}) and inhomogeneously broadened line widths between 5 and 7 nm (100−140 cm^{-1}). For P680, the optical reorganization energy is approximately 40 cm^{-1}. The half-bandwidth at room temperature is near 16 nm (338 cm^{-1}) and due to homogeneous and inhomogeneous components of 15 nm (324 cm^{-1}) and 5 nm (100 cm^{-1}), respectively. Thus, the room temperature bandwidth, in the case of all pigment sub-bands, is somewhat dominated by the homogeneous broadening.

In photosystem II of plants, photochemistry occurs at the reaction center where the primary donor (P680)[1] reduces the primary acceptor (pheophytin) with a time constant which is generally thought to be between 2 and 3 ps (Wasielewski et al., 1992; Roelofs et al., 1991). In recent years, it has been possible to isolate a relatively stable chl−protein complex which binds both the primary donor and acceptor in a functionally active state (Chapman et al., 1988). This complex, known as the D1/D2/cyt *b*-559 complex, binds in addition to P680 and pheophytin a number of accessory chlorin pigments. The most commonly reported chlorin stoichiometry is 6 chl and 2 pheophytin per RC complex, though a number of reports also suggest that 4 and 5 chl and 2 pheophytin preparations may also be achieved [for a review, see Seibert (1993) and Chang et al. (1994)]. Quite substantial differences in 4 K absorption spectra between preparations from different laboratories have also been reported, which may be correlated with the number of bound chls (Kwa et al., 1994; Chang et al., 1994). Thus, there is still no consensus as to the exact chlorin binding stoichiometry of the D1/D2/cyt *b*-559 complex in its native state.

The rather structureless Q_y absorption spectrum at RT is maximal at 676 nm and splits into two peaks near 670 and 679 nm at cryogenic temperatures (Tentenkin et al., 1989; Braun et al., 1990; Barber, 1992). The former peak is generally associated with accessory chlorins and the latter mainly with P680. In addition, some reports suggest the presence of a rather weak absorption shoulder around 683−684 nm in spectra measured at 4 K (Kwa et al., 1994; Chang et al., 1994). This long-wavelength structure is most evident in triplet-state bottleneck hole spectra, particularly when the burn wavelength is considerably to the red (Kwa et al., 1994; Chang et al., 1994). This red-absorbing pigment has been suggested to be particularly prominent in 6 chl/2 pheophytin preparations (Kwa et al., 1994). In addition, a number of reports using different spectroscopic techniques indicate that at 4 K the photoactive pheophytin lies spectroscopically very close to P680 (Tang et al., 1990; van der Vos et al., 1992), and this has been recently confirmed to also be the case at RT by light-induced bleaching of absorption spectra (Garlaschi et al., 1994). Thus, four pigment pools have been directly identified. Other attempts to characterize the absorption position of pigments have relied on spectral decomposition techniques (Tentenkin et al., 1989; van Kan et al., 1990; Garlaschi et al., 1994). In a recent report from this laboratory, a minimum fit of the Q_y region in terms of five Gaussian bands was presented for a 6 chl/2 pheophytin

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[1] Abbreviations: chl, chlorophyll, FWHM, full width at half-maximum; P680, primary electron donor of photosystem II; PSII, photosystem II; RC, reaction center; RT, room temperature.

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preparation, in which the presence of a pheophytin almost isoenergetic with P680 was described (Garlaschi et al., 1994). In addition, three sub-bands associated with accessory chlorins were suggested at shorter wavelengths. There are, however, several features of this sub-band description which are perhaps surprising and which therefore require further analysis. First, the sub-band associated with P680 is somewhat broader (23 nm at RT) than might be expected from hole burning data at 4 K (Tang et al., 1990) in which an optical reorganization energy of around 40 cm^{-1} for P680 was suggested together with an inhomogeneous bandwidth of around 100 cm^{-1}. Calculation suggests that this would give rise to a 15−16 nm (345 cm^{-1}) bandwidth at RT, which is in general agreement with the oxidized minus reduced P680 difference spectrum (Witt et al., 1986). However, the possibility also exists that the broad P680 decomposition band is associated with a very large sample inhomogeneity (Carbonera et al., 1994). A second unexpected feature of this description was the very low temperature sensitivity of the three accessory pigment sub-bands, possibly also associated with sample heterogeneity.

In order to specifically address the above-mentioned aspects and to throw more light on the absorption properties of the PSII−RC complex, we have undertaken an exhaustive thermal broadening absorption decomposition analysis between 80 and 300 K. As discussed below, this approach can, in principle, yield information on both the optical reorganization energy and the inhomogeneous broadening of pigment absorption bands. Thus, it is possible to directly compare absorption band characteristics with those obtained by hole burning and other line narrowing spectroscopies. In addition, this approach directly addresses the question of RT properties which are inaccessible by line narrowing techniques. The results, which are discussed in terms of permanent and transient hole burning data (Tang et al., 1990; Chang et al., 1994; Kwa et al., 1994), suggest a description in terms of seven main Gaussian sub-bands. All bands are inhomogeneously broadened between 100 and 150 cm^{-1}. The optical reorganization energy of the single P680 sub-band is around 40 cm^{-1} while this parameter for the other sub-bands is around 16 cm^{-1}.

MATERIALS AND METHODS

The D1/D2/cyt *b*-559 complex was obtained according to Chapman et al. (1988), starting from PSII membranes prepared from maize mesophyll chloroplasts (Bassi & Simpson, 1986) by the method of Berthold et al. (1981) as modified by Dunahay et al. (1984). The polypeptide composition was determined by SDS−polyacrylamide gel electrophoresis on a 12−17% gel containing 6 M urea and stained with Coomassie blue. The preparation was judged to be pure by comparison of the polypeptide pattern thus obtained with that reported by Barber et al. (1987). The absorption maximum at room temperature was close to 676 nm, which according to Booth et al. (1991) indicates that it had not undergone degradation. Pigment analysis indicates that the preparation used in the present study had a chlorophyll/pheophytin ratio of about 3 (Zucchelli et al., 1994), thus indicating that the preparation is of the 6 chlorophyll/2 pheophytin type.

Absorption spectra of the complex were measured using an EG&G OMAIII (Model 1460) with an intensified diode array (Model 1420) mounted on a spectrograph (Jobin-Yvon HR320) with a 150 groove mm^{-1} grating. The wavelength scale of the instrument was calibrated using a neon spectral calibration source (Cathodeon). The wavelength spacing between pixels is about 0.5 nm. Absorption was measured using light from a halogen lamp, attenuated by neutral filters (Balzers). The light path was 1 mm. Temperature regulation was achieved with a vacuum-assisted Joule-Thomson refrigerating system (Model K-2002T; MMR Technologies Inc.) for all spectra in the temperature range 80−300 K. The residual absorption at 750 nm was subtracted from the spectra. The sample was diluted to 0.15 OD mm^{-1} in a buffer containing 10 mM Hepes (pH 7.6), 0.02% dodecyl maltoside, and 64% glycerol.

Decomposition analysis of the spectra in terms of "asymmetric" Gaussian bands was performed as already described (Zucchelli et al., 1990) using a nonlinear least-squares algorithm that minimizes the χ^2 function with respect to the parameters of a model function. This model function is written as the sum of a variable number (maximum number 20) of "asymmetric" Gaussian curves. The number of bands used was initially fixed whereas all the other parameters defining each Gaussian curve (wavelength position, height, and two FWHM values) were free to move. In order to obtain a "minimum" band number description of a spectrum, the initial band positions chosen were typically more or less equidistant along the spectrum interval. If the model used is physically well founded for the system analyzed, a solution must exist, and it should be unique. However, in the case of extreme spectral congestion, from a numerical point of view the task is formidable. As each band has four independent parameters, the fit is performed over an hypersurface in a multidimensional space in which the search for a minimum could be complicated by the presence of more than one minimum, particularly in the presence of high sub-band congestion. In the present study, this was particularly the case for the six and seven sub-band description. It was thus necessary to implement the numerical procedure with additional information and constraints. This was possible as the decomposition program used allows modification of parameter values at the error minimum. In this way, we were able to include in the minimum five sub-band's description an extra one or two bands (six or seven band description) as described under Results.

RESULTS

The D1/D2/cyt *b*-559 complex used in this analysis is from the same preparation as that previously used (Garlaschi et al., 1994; Zucchelli et al., 1995) and is of the 6 chl/2 pheophytin type (Zucchelli et al., 1995). It was demonstrated that the fluorescence is from an almost perfectly equilibrated state in which uncoupled pigments could not be detected (Zucchelli et al., 1995). The RT and 10 K absorption spectra are shown in Figure 1. At RT, the maximum is at 676 nm while at 10 K two clearly resolved peaks near 670 and 679 nm are visible. In general terms, these characteristics are similar to other published spectra for this complex. It should be pointed out, however, that considerably variation in detailed absorption properties is encountered in preparations from different laboratories (Kwa et al., 1994; A. Holzwarth, personal communication). Recently Chang et al. (1994) have correlated these differences with different chl/pheophytin stoichiometries. According to these authors, maximum

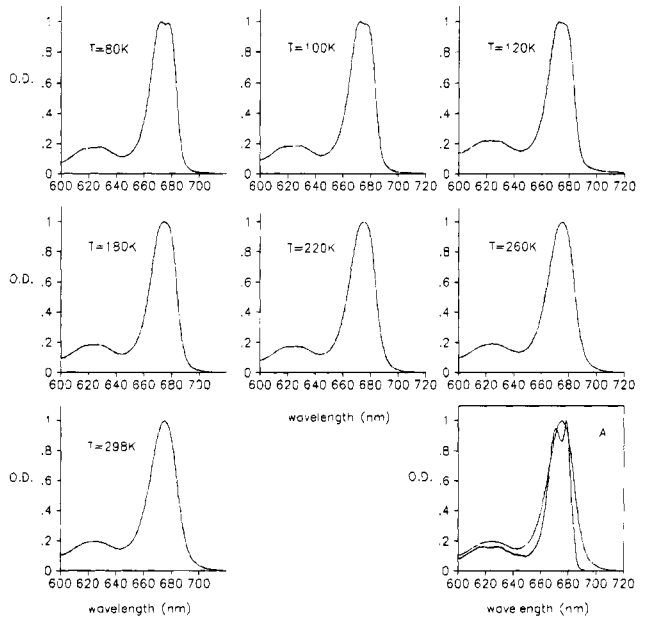

FIGURE 1: Absorption spectra between 80 and 300 K of the D1/D2/cyt *b*-559 complex. Comparison between the absorption spectra measured at room temperature and 10 K.

resolution of the 670 and 679 nm peaks is associated with the 4 chl/2 pheophytin preparation, while the 6 chl/2 pheophytin complex displays less well-resolved peaks. We would point out, however, that the present preparation shows extremely well-resolved peaks at 10 K, similar to those of the 4 chl/2 pheophytin complex of Chang et al. (1994) even though the chl/pheophytin stoichiometry is 6/2.

As other PSII chl−protein complexes have absorption maxima in the 674−676 nm range (Jennings et al., 1994), the presence of the pronounced minimum at 675 nm clearly rules out the presence of significant contamination with other pigment binding complexes. Our previous demonstration of thermal equilibration in this preparation (Zucchelli et al., 1995) is also in line with this conclusion.

In the following, we have analyzed the thermal broadening of pigment absorption sub-bands in terms of a model in which excited-state electrons are quite strongly coupled to a single phonon frequency. This model is derived from the extensive hole burning studies of chl−protein complexes of Small and collaborators (Hayes et al., 1988). In this hypothesis, the half-bandwidth of the homogeneously broadened pigment absorption ($FWHM_{hom}$, cm^{-1}) is given by

$$FWHM^2_{hom} = 8\ln(2)S\nu^2_m \coth\left(\frac{h\nu_m c}{2kT}\right)$$

where S is the Huang−Rhys coupling strength, ν_m (cm^{-1}) is the mean phonon frequency associated with protein vibrations, c is the velocity of light, h is the Planck constant, k is the Boltzmann constant, and T is the absolute temperature.

For chl−protein complexes, including the D1/D2/cyt *b*-559 complex, ν_m has been determined to have a value close to 20 cm^{-1}. Thus, for the temperature range 70−300 K, this expression may be approximated by

$$FWHM^2_{hom} \cong 16\ln(2)\frac{k}{hc}S\nu_m T$$

In addition to homogeneous broadening, pigment spectra in chl−protein complexes are also subject to the inhomogeneous broadening ($FWHM_{inh}$) associated with statistical fluctuations in the binding site which lead to a statistical distribution of absorption energies. Inhomogeneous broadening is generally assumed to be independent of temperature and is frozen in at low temperatures. Thus, the overall FWHM for Gaussian absorption bands is given by

$$FWHM^2 = FWHM^2_{hom} + FWHM^2_{inh}$$

Therefore, from an analysis of the FWHM as a function of temperature, it is possible to determine the optical reorganization energy ($S\nu_m$) and the $FWHM_{inh}$ of the pigment absorption band.

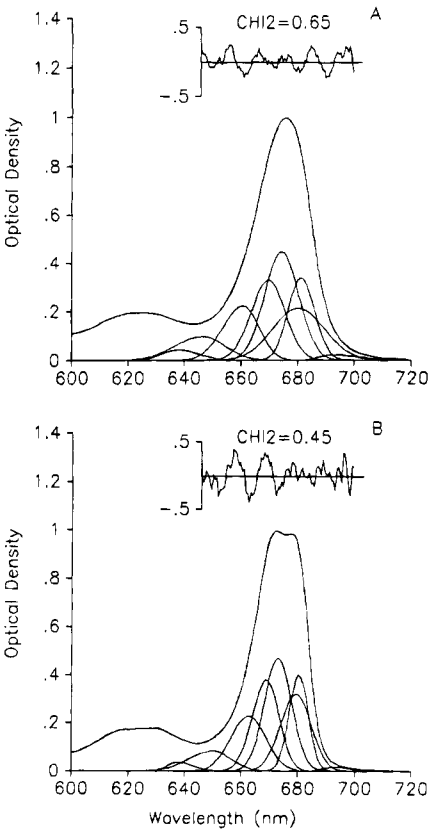

FIGURE 2: Gaussian five sub-band decomposition description of D1/D2/cyt *b*-559 absorption spectra measured at room temperature (A) and 80 K (B). The residuals are the differences between measured and calculated spectra weighted by the errors of the optical density measurements. The reduced χ^2 values are also shown. The spectrum presented is that measured which on this scale is indistinguishable from that calculated as the sum of the decomposition bands.

The thermal broadening analysis will be presented for three different decomposition descriptions which will be presented and discussed separately. The spectra used in this analysis are presented in Figure 1. As mentioned under Discussion, the Gaussian model is expected to be appropriate in the 80−300 K temperature range.

(a) Five Sub-band Description. This is shown in Figure 2 and Table 1 and is similar to that presented previously (Garlaschi et al., 1994) in which the shorter wavelength region of accessory pigments is described by three broad Gaussians. The red region is fitted by two sub-bands, the narrower of which is pheophytin and the broader is P680, as discussed previously (Garlaschi et al., 1994). The thermal broadening analysis has been performed for all sub-bands with the exception of that at approximately 660 nm (Figure

Table 1: Gaussian Parameters for the Five Sub-band Decomposition of Absorption Spectra of the D1/D2/Cyt *b*-559 Complex Measured between 80 and 300 K

description a	80 K	100 K	140 K	180 K	220 K	260 K	RT
band I							
λ_{max} (nm)	663.2	663.0	662.6	662.1	661.9	661.7	661.3
FWHM (nm)	14.5	15.0	14.7	14.9	14.9	15.1	15.0
	7.2 7.3	7.3 7.7	7.5 7.2	7.7 7.2	8.0 6.9	7.9 7.2	8.3 6.7
area (%)[a]	16.6	16.9	16.3	15.7	15.3	15.2	14.7
band II							
λ_{max} (nm)	669.5	669.6	669.8	669.9	670.4	670.3	670.2
FWHM (nm)	11.2	11.4	11.9	12.7	12.9	13.3	14.2
	5.8 5.4	5.9 5.5	6.2 5.7	6.5 6.2	6.7 6.3	7.0 6.3	7.2 7.0
area (%)	21.1	20.8	20.7	20.8	20.6	20.3	20.6
band III							
λ_{max} (nm)	673.6	673.6	673.8	673.9	674.4	674.6	674.8
FWHM (nm)	11.4	11.8	12.3	12.7	13.2	13.5	14.2
	5.6 5.8	5.8 6.0	6.0 6.3	6.1 6.6	6.7 6.5	6.8 6.7	7.0 7.2
area (%)	26.6	26.4	26.7	26.9	26.8	26.8	27.9
band IV							
λ_{max} (nm)	680.0	680.0	679.6	679.6	680.0	680.6	680.8
FWHM (nm)	12.5	13.6	15.1	17.1	18.6	20.3	21.6
	6.3 6.2	6.8 6.8	7.4 7.7	8.3 8.8	9.4 9.2	9.8 10.5	10.7 10.9
area (%)	20.0	20.0	20.0	20.0	20.9	21.3	20.4
band V							
λ_{max} (nm)	680.9	681.0	681.2	681.2	681.3	681.4	681.7
FWHM (nm)	7.9	8.1	8.5	9.0	9.4	10.1	11.0
	4.0 3.9	4.1 4.0	4.3 4.2	4.5 4.5	4.6 4.8	5.0 5.1	5.3 5.7
area (%)	15.7	15.9	16.3	16.0	16.4	16.5	16.4

[a] The percentage areas of the spectral forms have been calculated from the total area given by the sum of all the bands. The FWHM is given as the left and right values, together with the sum of these two values.

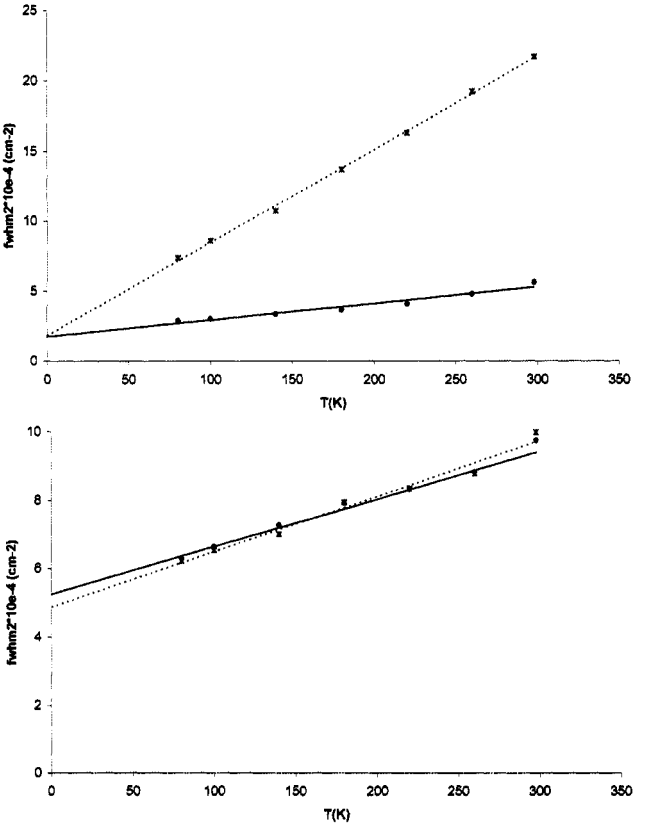

FIGURE 3: Square of the FWHM as a function of temperature for the five sub-band description of the D1/D2/cyt *b*-559 absorption spectra. (A) P680 (★) and pheophytin (●). (B) Sub-band 670 nm (★) and sub-band 674 nm (●). The straight lines are the linear regresson fits with $r = 0.99$.

3, Table 2). This is because in the wavelength range below 660 nm contributions due to the different intramolecular vibrational modes of chlorophyll *a* are expected to become significant (Gillie et al., 1989). The lowermost vibrational modes have frequencies in the range of about 260−500 cm^{-1} (Gillie et al., 1989) so that their contribution is around 660−670 nm for the redmost bands. However, the coupling of these frequencies is very weak (Gillie et al., 1989), and they are therefore unlikely to lead to significant absorption contributions. For example, using the Franck−Condon factors (Champion & Albrecht, 1980), a total contribution of about 5% for the vibrational transitions between 260 and 500 cm^{-1} with respect to the zero−zero transition is obtained. As the absorption intensity associated with these vibrational modes is distributed over the spectral region (660−670 nm) described by the shorter wavelength sub-bands, we estimate that this contribution should not exceed about 2.5% for any of these bands. Higher vibrational frequencies (500−1500 cm^{-1}) fall outside the spectral interval of present interest. In the spectral decompositions, the two shorter wavelength bands represented are considered to be associated with some of these vibrational modes for the different spectral forms. These comments are valid for the six and seven sub-band descriptions presented below.

Table 2: Optical Reorganization Energy ($S\nu_m$) and $FWHM_{inh}$ for Gaussian Sub-bands from Thermal Broadening Analysis of the Five Sub-band Description

description a	$S\nu_m$ (cm^{-1})	$FWHM_{inh}$ (cm^{-1})
P680	86	135
Pheo	17	133
674 nm	18	229
670 nm	21	220

It is clear that in all cases the data can be reasonably represented by a linear approximation. The band which is in closest agreement with literature values (Tang et al., 1990) is the pheophytin band near 681 nm ($S\nu_m$ = 17 cm^{-1}, $FWHM_{inh}$ = 133 cm^{-1}). The broad P680 band (FWHM = 22 nm at RT) yields an $S\nu_m$ value ($S\nu_m$ = 85 cm^{-1})

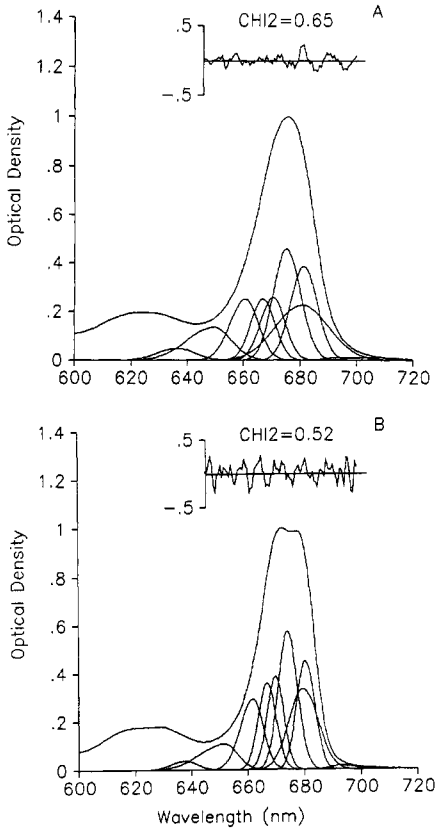

FIGURE 4: Gaussian six sub-band decomposition description of D1/D2/cyt *b*-559 absorption spectra measured at room temperature (A) and 80 K (B). See legend to Figure 2 for all other details.

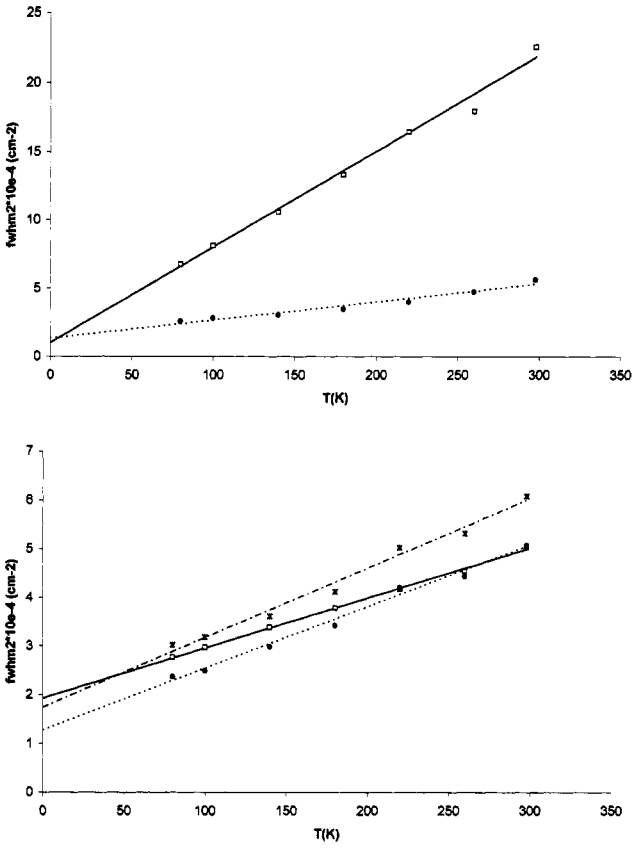

FIGURE 5: Square of the FWHM as a function of temperature of the six sub-band description of the D1/D2/cyt *b*-559 absorption spectra. (A) P680 (□) and pheophytin (●). (B) Sub-band 675 nm (★), sub-band 670 nm (●), and sub-band 667 nm (□). The straight lines are linear regression fits with $r = 0.98-0.99$.

approximately double that coming from hole burning (Tang et al., 1990; Chang et al., 1994). In addition, the oxidized minus reduced difference absorption difference spectrum of P680 suggests an approximately 16 nm bandwidth at RT (Witt et al., 1986). The two accessory pigment bands analyzed yield $FWHM_{inh}$ values around 230 cm^{-1} which are considerably greater than those of the red bands and are also greater than those of antenna chls in LHCII (Reddy et al., 1994). We have therefore concluded that this description is not entirely satisfactory and searched for others.

(b) Six Sub-band Description. This is shown in Figure 4 and Table 3. Here we have addressed the problem of what appears to be the excessively large inhomogeneous broadening ascribed in the five sub-band description to the accessory pigment bands. To this end, a fourth Gaussian has been included in the shorter wavelength of the $Q_y(0,0)$ region, leaving the two redmost sub-bands approximately as in description (a). The wavelength positions of the four accessory pigment sub-bands come out near 661, 667, 670, and 675 nm. With the exception of the fourth accessory pigment band near 667 nm, the wavelength positions of all other sub-bands are similar to those in (a). An important difference, however, is that inclusion of the fourth sub-band in this spectral region produced a pronounced narrowing of all accessory pigment sub-bands. The $S\nu_m$ values of the three accessory pigments analyzed (Figure 5, Table 4) are between 13 and 18 cm^{-1}, and $FWHM_{inh}$ values are in the range 110–130 cm^{-1} for the 670 and 675 nm sub-bands. These values are in rather good agreement with data from hole burning on nonphotochemically active antenna pigments (Reddy et al., 1994).

Inclusion of the fourth accessory pigment sub-band did not greatly modify the two red sub-bands, representing pheophytin and P680, which have respectively $S\nu_m$ values of 17 and 91 cm^{-1} and $FWHM_{inh}$ values of 115 and 100 cm^{-1}. Thus, the six sub-band description, apart from providing an excellent description of the D1/D2/cyt *b*-559 absorption, is in good agreement with literature values with the single exception of the rather broad P680 band associated with a high apparent optical reorganization energy ($S\nu_m$).

(c) Seven Sub-band Description. This is shown in Figure 6 and Table 5. Here we have examined the red side of the absorption spectrum, specifically addressing the high $S\nu_m$ values (80–90 cm^{-1}) hitherto found here for P680, which is double that suggested by hole burning studies on this complex at 4 K (Tang et al., 1990; Chang et al., 1994). To this end, we have sought a fit in the 80–300 K temperature interval in which an $S\nu_m$ value of approximately 40 cm^{-1} and an FWHM of about 100 cm^{-1} were imposed on the P680 band (Figure 7, Table 6). The exact values for these parameters were not fixed, but the fit was "encouraged" in this direction. All other parameters were allowed to fluctuate around values obtained in the previous description (b). In this way, it has been found that a good sub-band description is possible only with the inclusion of a major additional red sub-band with an absorption maximum near 683–684 nm. This conclusion is extremely interesting as evidence has very recently been presented, from triplet minus singlet spectra (Chang et al., 1994; Kwa et al., 1994), for the presence of a pigment absorbing near these wavelengths in some D1/D2/cyt *b*-559 preparations. We wish to emphasize that in order

Table 3: Gaussian Parameters for the Six Sub-band Decomposition of Absorption Spectra of the D1/D2/Cyt *b*-559 Complex Measured between 80 and 300 K[a]

description b	80 K	100 K	140 K	180 K	220 K	260 K	RT
band I							
λ_{max} (nm)	662.5	662.5	662.2	662.1	661.9	661.8	661.3
FWHM (nm)	9.6	9.7	10.0	10.4	10.6	10.9	11.8
	5.4 4.2	5.3 4.4	5.4 4.6	5.6 4.8	5.7 4.9	5.9 5.0	6.3 5.5
area (%)	14.0	14.0	13.6	13.3	12.8	12.8	13.2
band II							
λ_{max} (nm)	667.6	667.6	667.7	667.7	667.8	667.7	667.5
FWHM (nm)	7.5	7.6	8.1	8.7	9.2	9.5	10.0
	3.8 3.7	3.8 3.8	4.1 4.0	4.4 4.3	4.6 4.6	4.8 4.7	5.0 5.0
area (%)	13.3	13.0	12.6	12.2	11.9	11.6	11.2
band III							
λ_{max} (nm)	670.5	670.6	670.5	670.5	670.6	670.6	671.1
FWHM (nm)	6.9	7.1	7.8	8.4	9.2	9.5	10.1
	3.6 3.3	3.7 3.4	4.0 3.8	4.3 4.1	4.5 4.7	4.7 4.8	5.1 5.0
area (%)	13.3	12.7	12.5	12.1	12.2	12.3	11.6
band IV							
λ_{max} (nm)	674.8	674.8	675.0	675.1	675.3	675.3	675.7
FWHM (nm)	7.9	8.1	8.7	9.3	10.2	10.5	11.2
	3.9 4.0	4.0 4.1	4.2 4.5	4.5 4.8	4.7 5.5	4.9 5.6	5.3 5.9
area (%)	22.6	22.0	22.6	22.6	24.0	23.5	22.9
band V							
λ_{max} (nm)	680.2	680.1	679.8	679.8	680.2	680.6	681.2
FWHM (nm)	12.1	13.2	15.0	17.1	18.8	19.7	22.1
	5.9 6.2	6.5 6.7	7.4 7.6	8.3 8.8	9.4 9.4	9.4 10.3	10.9 11.2
area (%)	20.0	20.5	21.0	21.9	21.9	21.7	22.2
band VI							
λ_{max} (nm)	680.9	681.0	681.2	681.3	681.4	681.4	681.7
FWHM (nm)	7.4	7.8	8.0	8.7	9.3	10.1	11.0
	3.6 3.8	3.9 3.9	4.0 4.0	4.3 4.4	4.6 4.7	4.9 5.2	5.3 5.7
area (%)	16.8	17.7	17.8	18.0	17.1	18.1	19.0

[a] For further details, see legend to Table 1.

Table 4: Optical Reorganization Energy ($S\nu_m$) and $FWHM_{inh}$ from Thermal Broadening Analysis of the Six Sub-band Description

description b	$S\nu_m$ (cm^{-1})	$FWHM_{inh}$ (cm^{-1})
P680	91	100
Pheo	17	115
675 nm	18	132
670 nm	16	113
667 nm	13	139

to attribute and $S\nu_m$ value of around 40 cm^{-1} and an $FWHM_{inh}$ near 100 cm^{-1} to P680 between 80 and 300 K, a significant 683−684 nm sub-band is absolutely necessary. The $S\nu_m$ and $FWHM_{inh}$ values of this redmost sub-band are near 16 and 124 cm^{-1}, respectively.

The relative band intensities may be used to suggest possible pigment stoichiometries in the assumption that extinction coefficients for the spectral forms are approximately equal and that the somewhat lower Q_y dipole strength of pheophytin with respect to chlorophyll *a* in organic solvents (Shipman, 1977) is maintained in the complex. Thus, for the 6 chlorophyll/2 pheophytin preparation, we suggest that the 662, 667, and 679 nm sub-bands each represent a single chlorophyll *a* monomer. The 675 nm sub-band could include both a chlorophyll and a pheophytin with the other pheophytin identified near 680 nm (Garlaschi et al., 1994). While the intensity of the P680 band varies at the different temperatures between 14.5% and 17.3%, our results are in closest agreement with a monomer intensity. The 683 nm sub-band, which has an intensity of around 10%, may be explained as a chlorophyll which is present in most, but not all, complexes present in the preparation.

In this description, a low intensity, rather broad Gaussian is present in the long-wavelength tail. This band increases in intensity in parallel with the decomposition narrowing of the P680 sub-band. We do not know if this represents a

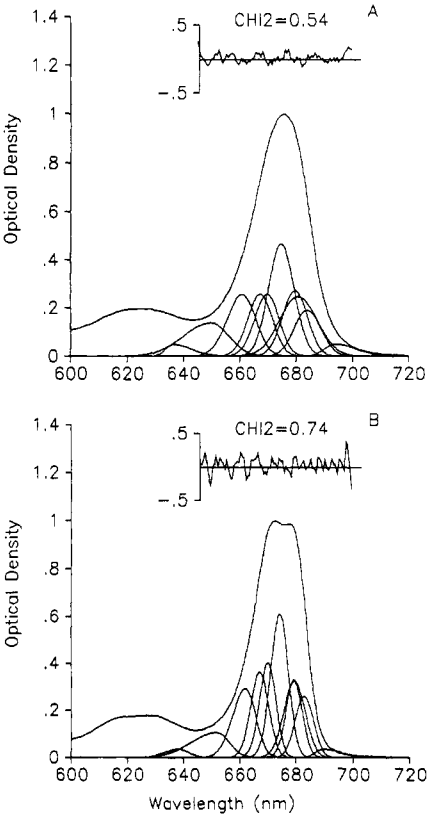

FIGURE 6: Seven gaussian sub-band decomposition description of D1/D2/cyt *b*-559 absorption spectra measured at room temperature (A) and 80 K (B). See legend to Figure 2 for all other details.

Table 5: Gaussian Parameters for the Seven Sub-band Decomposition of Absorption Spectra of the D1/D2/Cyt *b*-559 Complex Measured between 80 and 300 K[a]

description c	80 K	100 K	140 K	180 K	220 K	260 K	RT
band I							
λ_{max} (nm)	662.5	662.2	662.0	662.0	661.9	661.8	661.5
FWHM (nm)	9.7	9.9	10.1	10.5	10.6	11.0	11.9
	5.4 4.3	5.3 4.6	5.4 4.7	5.5 5.0	5.6 5.0	5.7 5.3	6.3 5.6
area (%)	14.3	14.0	14.0	13.8	13.1	13.4	13.9
band II							
λ_{max} (nm)	667.5	667.5	667.6	667.7	667.7	667.6	668.0
FWHM (nm)	7.5	7.9	8.3	9.0	9.3	9.8	10.4
	3.7 3.8	3.9 4.0	4.1 4.2	4.5 4.5	4.6 4.7	4.9 4.9	5.3 5.1
area (%)	13.8	13.9	13.5	13.2	12.8	12.7	12.1
band III							
λ_{max} (nm)	670.5	670.5	670.5	670.5	670.6	670.6	670.5
FWHM (nm)	6.9	7.3	7.9	8.6	9.2	9.5	10.2
	3.5 3.4	3.7 3.6	4.0 3.9	4.3 4.3	4.7 4.5	4.7 4.8	5.3 4.9
area (%)	13.8	13.8	13.3	13.1	12.9	12.2	12.0
band IV							
λ_{max} (nm)	674.6	674.6	674.6	674.6	674.7	674.8	675.3
FWHM (nm)	7.4	7.8	8.5	9.1	9.7	10.2	10.6
	3.7 3.7	3.9 3.9	4.2 4.3	4.4 4.7	4.8 4.9	5.0 5.2	5.3 5.3
area (%)	22.6	22.6	22.9	22.8	23.0	23.2	22.4
band V							
λ_{max} (nm)	680.0	680.2	680.2	680.4	680.5	680.8	681.3
FWHM (nm)	8.8	9.6	10.9	12.1	13.3	14.2	15.5
	4.4 4.4	4.8 4.8	5.5 5.4	6.1 6.0	6.5 6.8	7.0 7.2	7.6 7.9
area (%)	14.5	14.6	15.3	16.0	16.5	17.0	17.3
band VI							
λ_{max} (nm)	679.7	679.7	679.9	680.0	680.1	680.1	680.3
FWHM (nm)	6.7	7.1	7.7	8.4	9.0	9.5	10.2
	3.3 3.4	3.6 3.5	3.9 3.8	4.2 4.2	4.6 4.4	4.8 4.7	5.2 5.0
area (%)	11.2	11.3	11.3	11.5	11.9	11.6	12.7
band VII							
λ_{max} (nm)	683.4	683.3	683.2	683.0	682.9	683.3	684.3
FWHM (nm)	7.5	7.9	8.4	9.0	9.6	10.3	11.0
	3.7 3.8	3.9 4.0	4.2 4.2	4.4 4.6	4.6 5.0	5.0 5.3	5.2 5.8
area (%)	9.8	9.9	9.7	9.6	9.8	9.8	9.6

[a] For further detals, see legend to Table 1.

very red absorbing pigment in a small fraction of complexes in this preparation or whether it may be associated with temperature-activated, low-energy, vibrational transitions. This latter interpretation is supported by the apparent temperature sensitivity of this sub-band (Figure 7). Similar temperature-sensitive bands have been described in the long-wavelength absorption tails of antenna chl–protein complexes (Zucchelli et al., 1994).

The $S\nu_m$ and $FWHM_{inh}$ values for the pheophytin band are similar to the previous descriptions (a) and (b). For the shorter wavelength accessory pigments, the $S\nu_m$ and $FWHM_{inh}$ values are also little changed with respect to description (b).

DISCUSSION

In this paper, we present a thermal broadening analysis (80–300 K) of the D1/D2/cyt *b*-559 complex, for the $Q_y(0,0)$ absorption region, in terms of Gaussian decomposition sub-bands. It is important to emphasize that in this temperature range the Gaussian model is expected to be appropriate for describing pigment absorption bands as (1) homogeneously broadened site spectra will be dominated by the phonon side bands which are approximately Gaussian in shape (Osad'ko, 1979) and (2) the inhomogeneous site distribution function is expected to be Gaussian. A possible overlap of the homogeneously and inhomogeneously broadened $Q_y(0,0)$ transition with low-lying vibrational transitions is taken into account by the decomposition program used here which allows for Gaussian asymmetry. However, it should be mentioned that phonon coupling to the lowermost vibrational modes near 260 cm^{-1} is weak (Gillie et al., 1989). We estimate that this vibrational frequency will have an absorption intensity of only about 1% of the zero–zero transition.

In the Gaussian decomposition approach which we have used here, a numerically good fit between 80 and 300 K can be obtained with five main sub-bands. This represents the resolution limit of this technique, taken by itself, for the D1/D2/cyt *b*-559 complex. In order to provide a physically more accurate description, we have taken into account results from other laboratories, mainly using line narrowing spectroscopies. In this way, to the minimum five band description another two major sub-bands were included. We emphasize that the band positions of the minimum five band description were essentially unchanged in passing to the seven band description, as the additional two bands fell in relatively unoccupied spectral regions and had the effect of producing line narrowing. An important aspect of this approach is that it suggests a total absorption description of the D1/D2/cyt *b*-559 complex which is entirely consistent with most presently available spectroscopic evidence and which includes the description of several pigment absorptions which are not accessible to line narrowing spectroscopies. In addition, the RT absorption characteristics are described.

In the seven sub-band description, the short-wavelength side (≈655–675 nm), associated with accessory pigments, is described by four sub-bands with peak positions near 661,

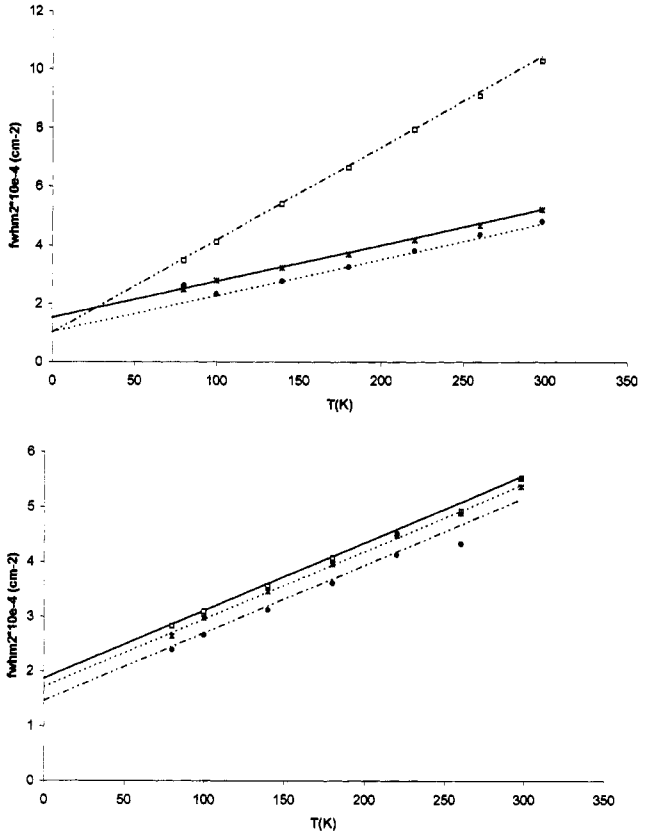

FIGURE 7: Square of the FWHM as a function of temperature for the seven sub-band description of the D1/D2/cyt *b*-559 absorption spectra. (A) P680 (□), pheophytin (●), and sub-band 684 nm (★). (B) Sub-band 674 nm (★), sub-band 670 nm (●), and sub-band 667 nm (□). The straight lines are linear regression fits at $r = 0.99$.

667, 670, and 675 nm. The optical reorganization energy ($S\nu_m$) of the latter three bands comes out near 10−20 cm^{-1} (0.5−1 nm), in agreement with the hole burning of antenna chlorophylls bound to LHCII (Reddy et al., 1994). The inhomogeneous line widths ($FWHM_{inh}$) are between 120 and 150 cm^{-1}, with the latter, slightly high value, which is associated with the 667 nm band, possibly being influenced by overlap with vibrational transitions of the redmost absorbing pigments. Thus, these $FWHM_{inh}$ values are quite reasonable and in line with antenna pigments. The half-bandwidths at room temperature are close to 11−12 nm (250−270 cm^{-1}). For a 12 nm (270 cm^{-1}) broad band in this wavelength range with a $FWHM_{inh}$ of 6 nm (140 cm^{-1}), one calculates an $FWHM_{hom}$ at RT of about 11 nm (232 cm^{-1}). Thus, the homogeneous broadening component is expected to be somewhat dominant with respect to the inhomogeneous one at RT. This conclusion is very similar to that suggested by a thermal broadening analysis of a long-wavelength pigment sub-band in the LHCII antenna complex of PSII (Jennings et al., 1994).

For the red side of the absorption spectrum, numerically good fits require the presence of two or three strongly overlapping sub-bands. The pheophytin and P680 bands are almost isoenergetic near 680 nm, in agreement with previous studies (Tang et al., 1990; van der Vos et al., 1992; Garlaschi et al., 1994). When two bands are used to fit the red region, the P680 sub-band is broad (about 22 nm at RT) and has an apparent $S\nu_m$ of 85−90 cm^{-1}, which is greatly in excess of that suggested by hole burning (Tang et al., 1990). While

Table 6: Optical Reorganization Energy ($S\nu_m$) and $FWHM_{inh}$ for Gaussian Sub-bands from Thermal Broadening Analysis of the Seven Sub-band Description

description c	$S\nu_m$ (cm^{-1})	$FWHM_{inh}$ (cm^{-1})
P680	43	95
Pheo	16	104
684 nm	16	122
674 nm	16	130
670 nm	16	117
667 nm	15	146

the high $S\nu_m$ value could be explained by a temperature effect on electron−phonon coupling, as hole burning was performed near 4 K, the broad sub-band is not in agreement with the approximately 16 nm bandwidth (RT) for the oxidized minus reduced difference spectrum of P680 (Witt et al., 1986). However, in the seven sub-band description, we show that it is possible to describe this spectral region between 80 and 300 K with a narrower P680 sub-band, having an $S\nu_m$ value which is in agreement with hole burning, if an additional long-wavelength sub-band is included. The absorption maximum for this red band is near 683−684 nm, which is in close agreement with the very recent results of Kwa et al. (1994) and Chang et al. (1994) where it was concluded that this transition represents a chl monomer which is not excitonically associated with P680. Thus, this sub-band is associated with an accessory chl, a conclusion which gains strong support from our observation that the $S\nu_m$ value is close to 20 cm^{-1}. The three red sub-bands have $FWHM_{inh}$ values in the 100−120 cm^{-1} (5−6 nm) range. These are in excellent agreement with hole burning for P680 and pheophytin, as are the $S\nu_m$ values for P680 (40 cm^{-1}) and pheophytin (16 cm^{-1}) (note that the $S\nu_m$ value of P680 in these decompositions was approximately fixed).

From this analysis, we conclude that the room temperature bandwidths are the following: P680, 16 nm (338 cm^{-1}); pheophytin, 10 nm (217 cm^{-1}); 684 nm chl, 12 nm (250 cm^{-1}). These values, together with the above $FWHM_{inh}$ values (100 cm^{-1}), yield homogeneously broadened bandwidths ($FWHM_{hom}$) at RT of the following: P680, 15 nm (324 cm^{-1}); pheophytin, 9 nm (186 cm^{-1}); 684 nm chl, 10 nm (217 cm^{-1}). Thus, as in the case of the shorter wavelength accessory pigments at RT, the homogeneous broadening is somewhat dominant. It is evident that the RT bandwidths are similar for all sub-bands except that associated with P680, which is considerably greater. This is due to a greater optical reorganization energy of this pigment, associated with stronger electron−phonon coupling (Tang et al., 1990; Chang et al., 1994).

The fluorescence Stokes shift for an absorption band, broadened only homogeneously, is given approximately by $2S\nu_m$. Thus, we conclude that for all D1/D2/cyt *b*-559 pigments except P680, this parameter has a value of approximately 1−2 nm (20−40 cm^{-1}). In the case of P680, the relevant value is about 4 nm (80 cm^{-1}). This is the correct value to consider in energy transfer studies at low temperatures where the inhomogeneous distribution is frozen in and each complex binds only one of each pigment type. However, at room temperature, if the dynamics of protein fluctuations are on the same time scale or faster than the excited-state lifetime, then energy will be equilibrated within the homogeneous distribution of each pigment site. In this case, for energy transfer the whole inhomogeneously broad-

ened absorption band needs to be considered. In the case of thermal equilibration within the inhomogeneous distribution, the Stokes shift is linear with FWHM (squared) (Kazachenko, 1965). This means that in the case of P680 there is almost no difference between the Stokes shift for the purely homogeneously broadened and total, inhomogeneously, broadened bands, with the latter being only about 10% greater than the former. However, in the case of the accessory pigments and the pheophytin absorbing near 680 nm, the Stokes shift of the total inhomogeneously broadened band is about 35% greater than that of the purely homogeneously band, which puts it in the range 2−3 nm. These values for the Stokes shift of inhomogeneously broadened accessory pigment absorption bands are very similar to those previously suggested for a number of LHCII spectral forms (Zucchelli et al., 1992).

The present paper confirms recently published observations concerning the presence of an accessory chl absorbing to the red of P680 (Chang et al., 1994; Kwa et al., 1994) in the D1/D2/cyt *b*-559 complex. It is therefore interesting to speculate on its function. Chang et al. (1994), on the basis of detergent sensitivity, suggested that it might be a "linker" chl in excitation transfer from core antenna complexes to the RC complex. In the triplet minus singlet spectra of Kwa et al. (1994), excited at 691 nm, the 684 nm band was shown to be extremely broad at 4 K (about 9.5 nm or 200 cm^{-1}). This is much broader than would be expected from the $FWHM_{inh}$ of about 100 cm^{-1} reported here. It therefore seems reasonable to conclude that excitation in the low-energy tails of the zero phonon lines of the 684 nm chl at 691 nm may have resulted in triplet transfer to some P680 molecules, in particular to those in the lower energy part of their inhomogeneous distribution. This suggestion can explain the broad bandwidth. If this is correct, it would indicate that P680 and the red pigment are extremely close, within van der Waals contact distance, as this is necessary for triplet transfer via the exchange mechanism (Dexter, 1953). This possibility is extremely interesting as it has been recently suggested (Fischer & Hoff, 1992) that red pigments can increase the energy trapping rates only when they are energetically not much lower than the primary donor. This is the case for the 684 nm chl which is about 0.4 kT (RT) below P680. Thus, this pigment could have the function of focusing energy transfer on the primary donor in the PSII RC complex.

We have recently suggested that all PSII antenna complexes contain significant amounts of a chl absorbing near 684 nm (Jennings et al., 1993; Zucchelli et al., 1994). For the four outer antenna complexes (LHCII, CP24, CP26, CP29), these transitions display a marked temperature sensitivity (Zucchelli et al., 1994), which however seems not to be the case for CP47 (Chang et al., 1994). The long-wavelength pigment in the D1/D2/cyt *b*-559 complex is also not temperature-sensitive. Thus, it seems that the pigment/protein or pigment/pigment interactions giving rise to the 684 nm absorption bands are different in different complexes.

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REFERENCES

Barber, J. (1992) *Photosynthetica 27*, 63−80.
Barber, J., Chagman, D. J., & Telfer, A. (1987) *FEBS Lett. 220*, 67−73.
Bassi, R., & Simpson, D. J. (1986) *Carlsberg Res. Commun. 51*, 363−370.
Berthold, D. A., Babcock, V. T., & Yocum, C. F. (1981) *FEBS Lett. 134*, 231−234.
Booth, P. J., Crystall, B., Ahmed, I., Barber, J., Porter, G., & Klug, D. R. (1991) *Biochemistry 30*, 7573−7586.
Braun, P., Greenberg, B. M., & Schatz, A. (1990) *Biochemistry 29*, 10376−10387.
Carbonera, G., Di Valentin, M., Giacometti, G., & Agostini, G. (1994) *Biochim. Biophys. Acta 1185*, 167−176.
Champion, P. M., & Albrecht, A. C. (1980) *J. Chem. Phys. 72*, 6498−6506.
Chang, H. C., Jankowiak, R., Reddy, N. R. S., Yocum, C. F., Picorel, R., Seibert, M., & Small, G. J. (1994) *J. Phys. Chem. 98*, 7725−7735.
Chapman, D. J., Gounaris, K., & Barber, J. (1988) *Biochim. Biophys. Acta 933*, 423−431.
Dexter, D. L. (1953) *J. Chem. Phys. 21*, 836−850.
Dunahay, T. G., Staehelin, L. A., Seibert, M., Ogilvie, P. D., & Berg, S. P. (1984) *Biochim. Biophys. Acta 764*, 179−193.
Fischer, M. R., & Hoff, A. J. (1992) *Biophys. J. 63*, 911−916.
Garlaschi, F. M., Zucchelli, G., Giavazzi, P., & Jennings, R. C. (1994) *Photosynth. Res. 41*, 465−473.
Gillie, J. K., Small, G. J., & Golbeck, J. H. (1989) *J. Phys. Chem. 93*, 1620−1627.
Hayes, J. M., Gillie, J. K., Tang, D., & Small, G. T. (1988) *Biochim. Biophys. Acta 932*, 287−305.
Jennings, R. C., Bassi, R., Garlaschi, F. M., Dainese, P., & Zucchelli, G. (1993) *Biochemistry 32*, 3203−3210.
Jennings, R. C., Garlaschi, F. M., Finzi, L., & Zucchelli, G. (1994) in *Proceedings of International Workshop on Pigment Protein Complexes: Structure and Spectral Properties*; Special Issue of *Lith. J. Phys. 34*, 293−300.
Kazachenko, L. P. (1965) *Opt. Spectrosc. 18*, 397−398.
Kwa, S. L. S., Eijckelhoff, C., van Grondelle, R., & Dekker, J. P. (1994) *J. Phys. Chem. 98*, 7702−7711.
Osad'ko, I. S. (1979) *Sov. Phys. Usp. 22*, 311−327.
Reddy, N. R. S., van Amerongen, H., Kwa, S. L. S., van Grondelle, R., & Small, G. J. (1994) *J. Phys. Chem. 98*, 4729−4735.
Roelofs, T. A., Gilbert, M., Shuvalov, A., & Holzwarth, A. R. (1991) *Biochim. Biophys. Acta 1060*, 237−244.
Seibert, M. (1993) in *The Photosynthetic Reaction Center* (Deisenhofer, J., & Norris, J., Eds.) Vol. 1 p 317; Academic Press, New York.
Shipman, L. L. (1977) *Photochem. Photobiol. 26*, 287−292.
Tang, D., Jankowiak, R., Seibert, M., Yocum, C. F., & Small, G. J. (1990) *J. Phys. Chem. 94*, 6519−6522.
Tentenkin, V. I., Gulyaev, B. A., Seibert, M., & Rubin, A. B. (1989) *FEBS Lett. 250*, 459−463.
van Kan, P. T. M., Otte, S. C. M., Kleinherenbrink, F. A. M., Nieveen, M. C., Aartsma, T. J., & van Gorkom, J. H. (1990) *Biochim. Biophys. Acta 1020*, 146−152.
van der Vos, R., van Leeuwen, P. J., Braun, P., & Hoff, A. J. (1992) *Biochim. Biophys. Acta 1140*, 184−198.
Wasielewski, M. R., Johnson, D. G., Seibert, M., & Govindjee (1992) *Proc. Natl. Acad. Sci. U.S.A. 86*, 524−528.
Witt, H. T., Schlodder, E., Brettel, K., & Saygin, O. (1986) *Ber. Bunsen-Ges. Phys. Chem. 90*, 1015−1024.
Zucchelli, G., Jennings, R. C., & Garlaschi, F. M. (1990) *J. Photochem. Photobiol. B: Biol. 6*, 381−394.
Zucchelli, G., Jennings, R. C., & Garlaschi, F. M. (1992) *Biochim. Biophys. Acta 1099*, 163−169.
Zucchelli, G., Dainese, P., Jennings, R. C., Breton, J., Garlaschi, F. M., & Bassi, R. (1994) *Biochemistry 33*, 8982−8990.
Zucchelli, G., Garlaschi, F. M., Croce, R., Bassi, R., & Jennings, R. C. (1995) *Biochim. Biophys. Acta 1229*, 59−63.

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