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Thermal Behavior of Long Wavelength Absorption Transitions in *Spirulina platensis* Photosystem I Trimers

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ABSTRACT In photosystem I trimers of *Spirulina platensis* a major long wavelength transition is irreversibly bleached by illumination with high-intensity white light. The photobleaching hole, identified by both absorption and circular dichroism spectroscopies, is interpreted as the inhomogeneously broadened Q_y transition of a chlorophyll form that absorbs maximally near 709 nm at room temperature. Analysis of the mean square deviation of the photobleaching hole between 80 and 300 K, in the linear electron-phonon frame, indicates that the optical reorganization energy is 52 cm^{-1}, four times greater than that for the bulk, short-wavelength-absorbing chlorophylls, and the inhomogenous site distribution bandwidth is close to 150 cm^{-1}. The room temperature bandwidth, close to 18.5 nm, is dominated by thermal (homogeneous) broadening. Photobleaching induces correlated circular dichroism changes, of opposite sign, at 709 and 670 nm, which suggests that the long wavelength transition may be a low energy excitonic band, in agreement with its high reorganization energy. Clear identification of the 709-nm spectral form was used in developing a Gaussian description of the long wavelength absorption tail by analyzing the changing band shape during photobleaching using a global decomposition procedure. Additional absorption states near 720, 733, and 743 nm were thus identified. The lowest energy state at 743 nm is present in substoichiometric levels at room temperature and its presence was confirmed by fluorescence spectroscopy. This state displays an unusual increase in intensity upon lowering the temperature, which is successfully described by assuming the presence of low-lying, thermally populated states.

INTRODUCTION

The antenna of photosystem I (PSI) of both plants and cyanobacteria contain a large number of chlorophyll (Chl) molecules, most of which are characterized by having the S_1 electronic transition (Q_y) between 645 and 700 nm. In plants these Chls are distributed approximately evenly between a core complex, which also binds the primary electron donor P700 and a number of electron transfer cofactors, and eight light harvesting I (LHCI) complexes forming the so-called outer antenna (Jansson, 1994; Jennings et al., 1996; Jansson et al., 1997). In cyanobacteria it is only the core that binds Chl whereas the outer antenna is made up of phycobilin pigments. An extremely interesting property, common to all PSI, is the presence of a small number of red-absorbing Chl forms (<5% of total Chls), the Q_y transitions of which lie at lower energies than that of the primary donor P700. Although in plants most red forms are associated with the LHCI complexes (Croce et al., 1998), in cyanobacteria the core complexes have relatively high amounts (for recent review see Karapetyan et al., 1999).

The importance of the red Chl forms in understanding energy transfer from the antenna to P700 has been underlined in recent years both in core particles (Holzwarth et al., 1993; Hastings et al., 1994; Turconi et al., 1994) and in intact PSI-200 with its full antenna complement (Turconi et al., 1994; Croce et al., 1996, 2000), with most excitation energy being photochemically trapped only after transfer through the low-energy chlorophyll forms. Thus, relatively slow, thermally activated transfer processes are involved in energy diffusion to P700 and these have recently been suggested to constitute rate-limiting steps for primary photochemistry (Jennings et al., 1998; Croce et al., 2000), in agreement with earlier model calculations (Fischer and Hoff, 1992; Trissl, 1993; Jennings et al., 1997). Thus it seems unlikely that the red forms have a role in increasing the rate of photochemistry. On the other hand it is expected that they play an important role in light capture, particularly in the case of leaves or cells that are located underneath or inside vegetation systems in which the light environment is strongly influenced by light filtering and scattering (Anderson, 1986; Garlaschi et al., 1989; Koehne et al., 1999; Rivadossi et al., 1999). Rivadossi et al. (1999) have recently estimated that in certain natural light environments the red forms may account for as much as 20–30% of the total number of photons absorbed.

Despite the intriguing nature of these low-energy-absorbing chlorophylls, both from a biological and a physical point of view, little detail is known of their spectroscopic properties. This is largely due to the difficulty in identifying single absorption bands in the red wing of PSI due to considerable spectral overlap. It is evident, however, mainly on the basis of fluorescence measurements, that there is considerable spectral heterogeneity of red forms with emit-

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ting bands near 720, 730, and 740 nm for plants (Mullet et al., 1980; Wittmershaus, 1987; Mukerji and Sauer, 1990; Pålsson et al., 1995; Croce et al., 1996, 1998) and for cyanobacteria (Shubin et al., 1991, 1992; Trissl, 1993; Gobets et al., 1994; Karapetyan et al., 1997) in the 720–760-nm interval. Although direct evidence for the wavelength positions of the absorption origin bands of these emitting species is lacking, absorption forms near 695, 708, and 716 nm have been tentatively identified in plants (e.g., Wittmershaus, 1987; Gobets et al., 1994), which, if correct, would suggest that they have very large Stokes' shifts, of the order of 10–25 nm. Similarly high Stokes' shift values have also been suggested for cyanobacterial red forms (Shubin et al., 1991; Gobets et al., 1994), although the 14-nm Stokes' shift of Gobets et al. has been recently contested (Rätsep et al., 2000; see Discussion). The very high values for the Stokes'shift reported for the chlorophyll red forms should be compared with those of normal antenna chlorophylls, which are of the order of 2 nm (Hayes et al., 1988; Zucchelli et al., 1996) and may indicate that the red forms have very unusual optical properties. This point was taken up in a thermal broadening analysis of the red absorption wing of plant PSI-200 and LHCI (Croce et al., 1998) in which it was concluded that the average optical reorganization energy ($S\nu_m$; where S is the electron-phonon coupling strength and ν_m is the mean phonon frequency) of all the low-energy forms analyzed together was in the range between 60 and 110 cm^{-1}, whereas a very recent hole-burning analysis of a chlorophyll red form in *Synechocystis* sp. (Rätsep et al., 2000) indicates a value of 80 cm^{-1}. These values are three to six times greater than for normal antenna chlorophylls (Hayes et al., 1988) and indicate that the average Stokes' shifts ($\sim 2S\nu_m$) for these red forms is in fact in the 6–12-nm range. In the present study we further analyze this point by the spectroscopic isolation by photobleaching of the absorption band of the dominant low-energy spectral form in trimers of the cyanobacterium *Spirulina platensis,* which has its absorption maximum near 709 nm (Jennings et al., 1998). The thermal broadening characteristics of this absorption band were analyzed both directly from the photobleaching difference spectra and also by a global Gaussian decomposition procedure. Both analyses yield an optical reorganization energy close to 52 cm^{-1}, indicative of strong electron-phonon coupling, and an inhomogeneous broadening (FWHM) in the 150–200-cm^{-1} interval. At room temperature the Q_y half bandwidth both in absorption and circular dichroism (CD) is close to 18 nm and is dominated by homogeneous broadening. To our knowledge this is the clearest spectroscopic characterization to date of a single red spectral form at room temperature, whereas a detailed low-temperature characterization of a red chlorophyll form was recently published (Rätsep et al., 2000).

An additional point of interest concerning the long-wavelength forms of PSI trimers of *Spirulina* is the presence of a strongly red-shifted fluorescence band (760 nm) at cryogenic temperatures but not at room temperature (Karapetyan et al., 1997). These authors suggested that this is associated with a chlorophyll form or state that absorbs near 746 nm. This form is present in PSI trimers and absent in monomers and it has been suggested to be caused by interactions between chlorophyll molecules on different subunits (Karapetyan et al., 1997, 1999). This state, the fluorescence yield of which displays a marked sensitivity to the redox state of P700, becomes detectable only at low temperatures. Subsequently, in a room temperature study of intact *Spirulina* cells, in which the red absorption tail was calculated from the emission spectra using the thermal equilibration assumption, Koehne and Trissl (1998) suggested that a long-wavelength absorption state, with maximum near 739 nm, may in fact be present at room temperature. In that study the temperature dependence was not investigated. In the present paper we have carefully studied this aspect using a global spectral decomposition approach. It is concluded that the red-most absorption state in PSI trimers of *Spirulina* is near 743 nm and that this is present at extremely low levels at room temperature, increasing in intensity in an approximately exponential manner as the temperature is lowered. The possible physical basis of this unusual thermal behavior is discussed.

MATERIALS AND METHODS

Experimental

Membranes from cells of the filamentous cyanobacterium *Spirulina platensis* were isolated using a French press and double centrifugation at 100,000 × *g* (1 h at 4°C) to remove phycobilisomes as described (Shubin et al., 1992). Membranes were treated with dodecyl β-D-maltoside (detergent/Chl ≈ 15), and PSI trimeric complexes were isolated using column chromatography on DEAE-Toyopearl according to Shubin et al. (1992). The Chl/P700 ratio in complexes was ~90 ± 5. PSI complexes were suspended in 50 mM Tris-HCL buffer (pH 8.0) and stored at 77 K until use. Samples, after thawing, were dissolved in 50 mM Tris-HCl buffer (pH 8.0).

Absorption and fluorescence spectra were measured with an OMA III (EG&G, model 1469, Parc, Princeton, CA) as described previously (Zucchelli et al. 1996). The wavelength spacing of the pixels was ~0.5 nm. Measurements were performed in the 70–300 K temperature range using a vacuum-assisted Joule-Thomson refrigerating system (model K-2002T, MMR Technology, Mountain View, CA) at a chlorophyll concentration of 2.5 μg/ml. For low-temperature measurements 65% glycerol (v/v) was present.

Circular dichroism spectra were measured in a Jasco-600 polarimeter with a bandwidth of 2 nm. Samples were routinely placed at 2 cm from the photomultiplier, and the optical density was 0.6 at the Q_y absorption maximum.

Photobleaching of *Spirulina platensis* trimers was performed using white light (34,000 μE m^{-2} s^{-1}) for between 40 and 90 min at 4°C.

Gaussian decompositions of absorption spectra were carried out by a global procedure in which a set of up to five spectra with different band shapes were simultaneously analyzed using a linear combination of up to 11 bands. The program searched for a solution using the same set of wavelength maxima and FWHM of the sub-band for all spectra whereas the amplitudes were completely free parameters. The goodness of the fit was determined by the χ^2 and residuals distribution.

Thermal band broadening analytical theory

For chlorophyll pigments bound to a protein the band-shape of the absorption transitions is mostly determined by two types of contribution. The first is due to coupling to low-frequency protein vibrational modes (phonons), which have been directly demonstrated to be in the 20–30-cm^{-1} range in a wide set of chlorophyll/protein complexes (Hayes et al., 1988). This is the dominant component of the so-called homogeneous broadening of the absorption band. The second is due to statistical fluctuations in the protein site energies and gives rise to the so-called site-inhomogeneous broadening, usually assumed to have a Gaussian distribution and to be temperature insensitive, although exceptions to this are known (Ormos et al., 1990). Thus, the mean square deviation (σ^2) of an absorption band of a particular pigment site, in the linear electron-phonon coupling assumption is given by (e.g., Hayes et al., 1988)

$$\sigma^2 = \sigma^2_{\text{hom}} + \sigma^2_{\text{inh}} = S\nu^2_{\text{m}}\coth[(hc\nu_{\text{m}})/(2k_{\text{B}}T)] + \sigma^2_{\text{inh}} \qquad (1)$$

where S and ν_{m} are the coupling strength and mean frequency (cm^{-1}) of the phonon bath, respectively, h is Planck's constant, c is the velocity of light, k_{B} is the Boltzmann constant, and T is the absolute temperature.

In the case of mean phonon frequencies in the 20–30-cm^{-1} range and for T = 70–300 K, Eq.1 simplifies to

$$\sigma^2 \approx 1.39S\nu_{\text{m}}T + \sigma^2_{\text{inh}} \qquad (2)$$

or

$$\text{FWHM}^2 \approx 7.7S\nu_{\text{m}}T + \text{FWHM}^2_{\text{inh}} \qquad (3)$$

in terms of the bandwidth parameter for Gaussian band shape. It is worth noting that for a composite absorption band with N different transitions an analogous expression can be written (Zucchelli et al., 1996):

$$\sigma^2_{\text{tot}} \approx 1.39 \sum_{\text{k}=1}^{\text{N}} A_{\text{k}}S_{\text{k}}\nu_{\text{mk}}T + \sigma^2_{\text{inh}_{\text{tot}}} \qquad (4)$$

where A_{k} is the area ($\Sigma_{\text{k}=1}^{\text{N}}A_{\text{k}} = 1$) of the kth absorption transition with coupling S_{k} to a mean phonon mode of frequency ν_{mk} (cm^{-1}).

From Eq. 2 it is evident that for a single absorption transition the plot of σ^2 versus T yields a straight line and from the slope and intercept the parameters $S\nu_{\text{m}}$ (excited-state reorganization energy) and σ_{inh} may be determined. In the case of a composite band (Eq. 4) the slope yields an average value for $S\nu_{\text{m}}$ of all underlying transitions and the intercept gives $\sigma_{\text{inh}_{\text{tot}}}$, which does not have a clear physical meaning as it contains contribution from both the site heterogeneity and site inhomogeneity.

RESULTS

Spectroscopic observations and photobleaching.

In Fig. 1 the absorption spectra for the chlorophyll Q_{y} region of *Spirulina* trimers, at several different temperatures, are presented together with the room temperature CD spectrum. Although the red tail at room temperature (RT) is rather structureless, at low temperatures two quite clearly defined bands emerge near 708 nm and 738 nm. The latter band was prominent in the 77 K spectrum of *Spirulina* trimers presented by Karapetyan et al. (1997) whereas the 709-nm structure was much less evident. This difference is probably due the use of the detergent Triton-X in the former study, which selectively destroys the 709-nm transition (unpublished observation). In the present investigation the milder detergent dodecylmaltoside was used. By inspection it is also evident that there is an increase in intensity in the red-most region of the absorption tail at low temperature, as previously observed (Karapetyan et al., 1997). In the RT CD spectrum, which has maxima at 670 nm (+) and 685 nm (−), a quite intense structure is also visible near 709 nm. This structure was absent from the previously published CD spectrum for trimers (Shubin et al.1992) due to its sensitivity to Triton-X (unpublished observation), the detergent used in that study. The CD spectrum goes to zero near 730 nm whereas the RT absorption extends further into the red by more than 20 nm. This indicates the presence of long-wavelength absorption forms or states that are not dichroic.

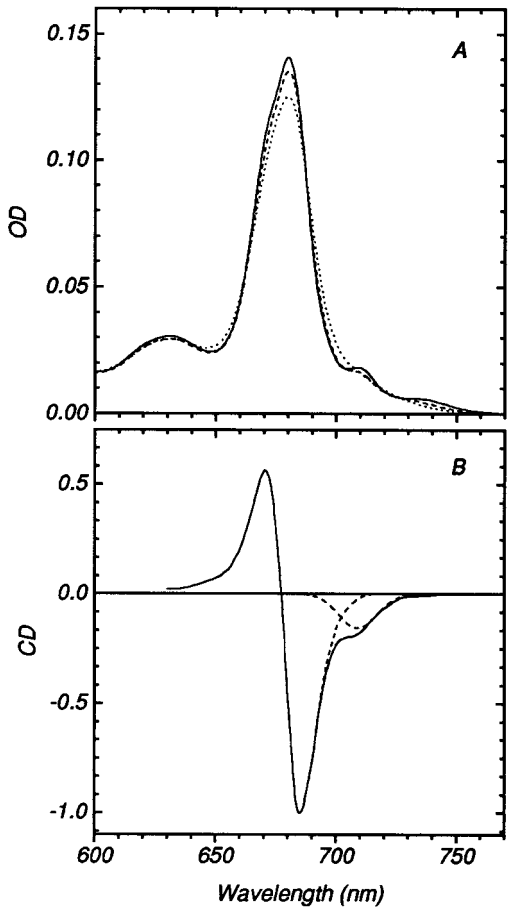

FIGURE 1 Absorption and circular dichroism spectra in the Q_{y} region of *Spirulina* photosystem I trimers. (*A*) Absorption spectra at 280 K (*dotted line*), 120 K (*dashed line*), and 80 K (*solid line*). (*B*) Circular dichroism spectrum at 280 K. Also shown is the Gaussian sub-band description of the negative lobe (*dashed line*). The CD signal was 35 millidegrees at the maximum value of the negative lobe.

It is well known that under high light intensities chlorophyll pigments bound to chlorophyll-protein complexes are subject to irreversible photobleaching and that this process is often differentially selective for different chlorophyll molecules (Garlaschi et al., 1994; Giuffra et al., 1997; Finzi

et al., 1998). We have noticed that when *Spirulina* trimers are exposed to high-intensity white light, photobleaching occurs over the entire spectral region analyzed, together with a small blue shift of the bulk pigment absorption band (Fig. 2 *A*). From the normalized spectra in Fig. 2 *A* it is, however, evident that the spectral region around 709 nm is selectively bleached. This selective bleaching around 709 nm is also evident in the CD spectra after normalization to the maximum of the negative lobe at 685 nm (Fig. 2 *b*). We emphasize the correctness of this normalization procedure as the band shape of the dominant 685-nm structure is little changed by photobleaching. A photobleaching-induced decrease in the CD signal of the positive lobe is also apparent that is similar in intensity to that of the 709-nm region.

To analyze the 709-nm absorption bleaching hole we have calculated the difference spectrum, after normalizing spectra to their maxima near 680 nm, over the temperature range 80–300 K. The bleaching maximum is at approximately 709 ± 1.0 nm at RT and appears to undergo a slight red shift of up to 1 nm as the temperature is lowered to 80 K. Several examples are presented in Fig. 3 where it can be seen that the bleaching hole is approximately symmetrical, within the errors, and can be reasonably well described by a Gaussian at each temperature. The baseline is taken as the relatively stable zero signal on the long wavelength side. There is some variability on the short wavelength side due to spectral overlap with the bulk pigments that also undergo photo-oxidation. We interpret this difference spectrum structure as representing the inhomogeneously broadened $Q_y(0, 0)$ transition of a single, approximately 709-nm-absorbing, chlorophyll form. The FWHM at room temperature is 18 ± 1.5 nm and 12 ± 0.5 nm at 100 K, where the errors represent the data spread. The conclusion that the photobleaching hole represents a single chlorophyll form is supported by the RT CD spectrum where the long wavelength signal is also well approximated by a single Gaussian, peaking near 709 nm, with a FWHM of 18.5 nm (Fig. 1 *B*).

The clear identification of some of the absorption characteristics of a major, long-wavelength-absorbing chlorophyll form in the red absorption wing of *Spirulina* trimers by both absorption and CD is useful for two reasons. First, it allows a direct thermal broadening analysis of its Q_y transition (Eq. 3) with the aim of determining the optical reorganization energy ($S\nu_m$) and the site inhomogeneous bandwidth ($FWHM_{inh}$). Second, it facilitates a decomposition description of the red absorption wing in terms of physically significant sub-bands.

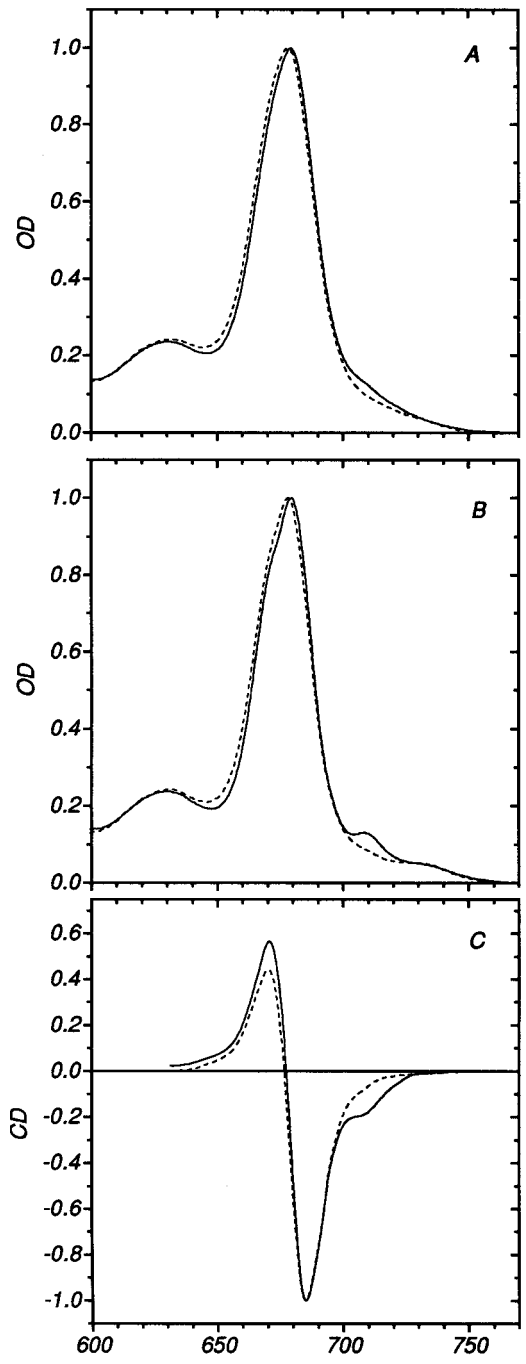

FIGURE 2 Effect of photobleaching on absorption and circular dichroism spectra of *Spirulina* photosystem I trimers in the Q_y region. (*A*) Absorption spectra at 270 K (*solid line*, control; *dashed line*, photobleached). (*B*) Absorption spectra at 120 K (*solid line*, control; *dashed line*, photobleached). (*C*) Circular dichroism spectra at 280 K (*solid line*, control; *dashed line*, photobleached). All spectra have been normalized to the maximum value of the main peak. The normalization factor was between 1.4 and 1.5 in all cases.

Thermal broadening analysis of the 709-nm photobleaching hole

As described in Materials and Methods (Eq. 2), the slope of the linear plot of σ^2 versus temperature yields the reorganization energy ($S\nu_m$) and the intercept gives the $\sigma_{inh}{}^2$. In Fig. 4 this plot is presented for the Gaussians used to describe the photobleaching hole at each temperature. Despite the considerable dispersion of the data above 250 K it is evident that a good linear fit is obtained yielding $S\nu_m$ =

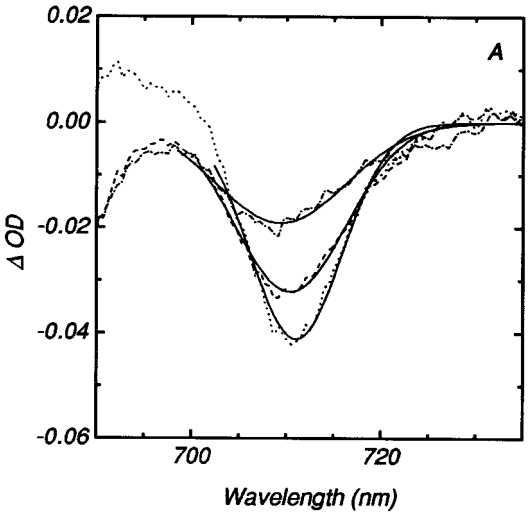

FIGURE 3 Photobleaching hole in the long wavelength absorption tail of *Spirulina* trimers at selected temperatures. Spectra were calculated as the photobleached samples minus control after normalization as shown in Fig. 2. Experimental difference spectra are shown by broken lines and the Gaussian approximations by solid lines for 280 K, 180 K, and 100 K.

52 cm^{-1} and $\sigma_{inh} = 64\ cm^{-1}$. This latter value is equivalent to a Gaussian $FWHM_{inh}$ of 150 cm^{-1}.

To compare the reorganization energy value with that of the non-red-shifted, bulk pigments of *Spirulina* trimers, we also present in Fig. 4 the thermal broadening plot for the composite band of the bulk chlorophylls. This band was defined as the trimer Q_y absorption from which the four long wavelength Gaussian sub-bands have been subtracted and for the RT spectrum is shown in Fig. 5. This is a composite band with absorption contributions from many different pigment forms, and the relevant analytical expression is given in Eq. 4. The average reorganization energy comes out as ~14 cm^{-1}, approximately four times less than for the 709-nm form.

Gaussian decomposition of the red absorption wing and thermal broadening analysis

Previous efforts at a sub-band description of the red absorption wing of *Spirulina* trimers have given varying results (Karapetyan et al., 1997; Koehne and Trissl, 1998). This indicates that from a purely numerical point of view many solutions exist. In an attempt to find a unique decomposition description that is also plausible from a physical point of view we have analyzed the changing band shape of the red absorption tail after bleaching by means of a global decomposition approach (see Materials and Methods) in which the presence of a transition peaking at 709 ± 0.5 nm with a FWHM at RT similar to that indicated in the above described difference spectrum analysis (18 ± 1.5 nm) was fixed. This is important as it orientates the sub-band description by providing a fixed reference with experimentally determined band shape characteristics. In the absence of this constraint many different numerically acceptable descriptions are possible, even with the global procedure. The remaining part of the red wing of the absorption spectrum

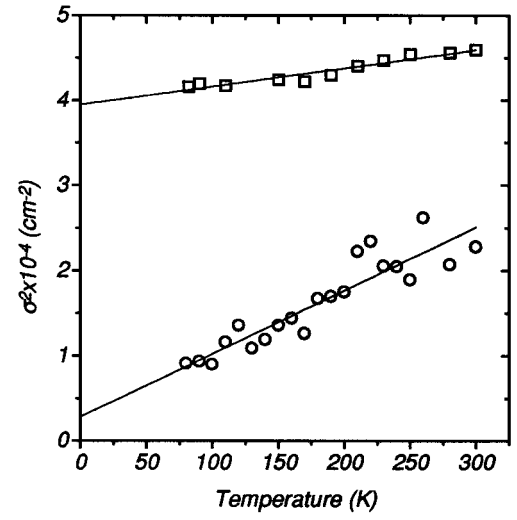

FIGURE 4 Mean square deviations (second order central moment; σ^2) of the 709-nm photobleaching hole (○) and the bulk chlorophyll band (□) between 80 and 300 K. For the photobleaching hole the σ^2 values were calculated using the Gaussian approximations as shown in Fig. 3. For the bulk chlorophyll band the σ^2 value refers to the band peaking at 680 nm shown in Fig. 5 (*dotted line*). The linear fits yield $S\nu_m = 52\ cm^{-1}$ and $\sigma_{inh} = 64\ cm^{-1}$ for the photobleaching hole and $S\nu_m = 14\ cm^{-1}$ for the bulk chlorophyll band.

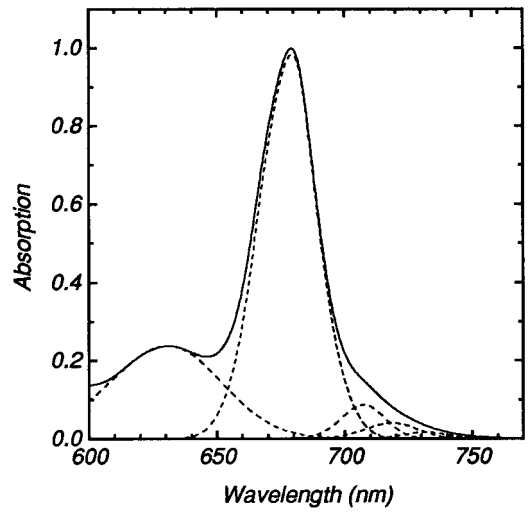

FIGURE 5 Gaussian sub-band description of the long wavelength absorption tail measured at room temperature. The global decomposition procedure used is described in the text. The bulk chlorophyll band (*dotted line*) is the linear combination of five Gaussians used to provide a purely numerical description of this absorption region. The long wavelength sub-band parameters are given in Table 1 together with those found when this description was propagated down to 80 K.

was described by a number of Gaussian bands whose parameters were free to vary. Photobleaching was assumed to change only the sub-band amplitudes. A minimum number of four sub-bands were found to fit the red wing at RT (Fig. 5 and Table 1) with maxima near 709, 720, 733, and 743 nm. When fewer sub-bands were used, the lowest-energy one becomes asymmetric toward the low-energy side, thus suggesting the requirement of at least another sub-band in this spectral region.

In the above sub-band description the area subtended by each of the first three sub-bands is significant whereas that associated with the red-most band is small and is clearly dependent on the baseline correction. To check this aspect we analyzed the fluorescence associated with the red absorption as this signal is intense. This was achieved by calculation of the fluorescence spectrum from the absorption spectrum using the Stepanov expression, Eq. 5 (Stepanov, 1957), as this has been shown to be accurate in describing the fluorescence of the red spectral forms in PSI (Croce et al., 1998; Pålsson et al., 1998). This expression assumes thermal equilibration of excited states between all energy levels in the system before fluorescence emission:

$$F(\nu)/A(\nu) \propto \nu^2 e^{-h\nu/k_BT} \quad (5)$$

Although there is a significant discrepancy between the calculated and measured spectra in the blue emission region, presumably due to the presence of some uncoupled pigments, the correspondence in the red emission region is excellent. In Fig. 6 we also present the calculated emission spectrum after subtraction of the 743-nm sub-band from the absorption spectrum. This leads to a significant blue shift in the long-wavelength emission band, not in agreement with measurement. We therefore conclude that a very low-intensity transition associated with the 743-nm sub-band is indeed present at RT in *Spirulina* trimers.

This sub-band description was then further tested by propagating it in the 80–270 K temperature range and the results of are presented in Table 1. In this analysis the wavelength positions of the sub-bands were fixed while the bandwidths and amplitudes were allowed to vary. For the 709-nm transition a linear σ^2 versus T plot was obtained (data not presented) yielding a value for $S\nu_m$ of $\sim$53 cm^{-1},

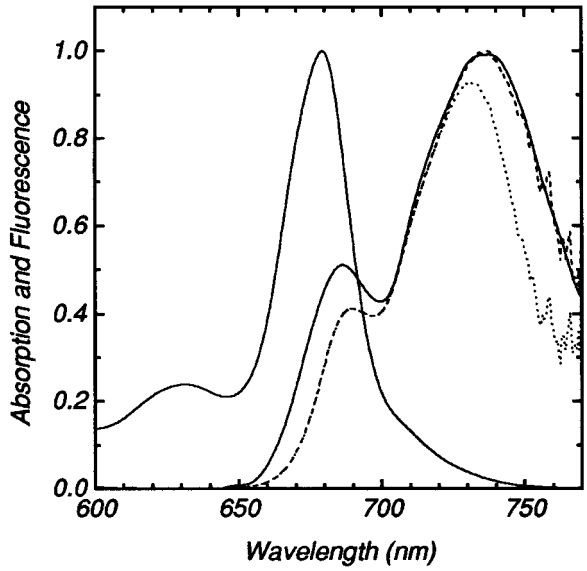

FIGURE 6 Comparison between the calculated (*dashed line*) and the measured steady-state fluorescence spectra (*solid line*) for photosystem I trimers of *Spirulina* at room temperature. The emission spectrum was calculated from the absorption spectrum, also shown in the figure, by the Stepanov equation (see text). The dotted-line fluorescence spectrum was calculated form the absorption spectrum in the same way but after subtraction of the minor, 743-nm absorption sub-band.

close to that determined from the difference spectrum analysis. For the longer-wavelength sub-bands the values of $S\nu_m$ fall in the interval between 60 and 100 cm^{-1} although, owing to fluctuations in the area of the red-most sub-bands, these results should be viewed with caution. The unusual thermal behavior of the red-most absorption region is largely described by the 743-nm sub-band, which increases approximately threefold in intensity as the temperature is lowered whereas the 709- and 720-nm sub-bands remain constant (Table 1). In Fig. 7 data are presented for the intensity change of the 743-nm sub-band from several different experiments. It is clear that the increase in intensity upon lowering the temperature is continuous and approximately exponential in shape. This result excludes the possibility that the changes in absorption intensity are asso-

TABLE 1 Gaussian parameters for the four-sub-band decomposition of the red absorption tail of *Spirulina* PSI trimers for selected temperatures between 80 and 300 K

	Parameter							
	Band 708.9 nm		Band 719.8 nm		Band 732.8 nm		Band 743.2 nm	
Temperature	FWHM (nm)	Area (%)	FWHM (nm)	Area (%)	FWHM (nm)	Area (%)	FWHM (nm)	Area (%)
80 K	11.9	5.2	14.9	3.0	13.5	1.8	14.8	1.3
150 K	14.7	5.3	19.6	3.4	17.6	2.0	17.6	1.0
200 K	16.8	5.2	22.3	3.5	20.1	1.9	22.9	0.5
250 K	17.9	5.4	24.9	3.6	22.2	1.6	25.6	0.4
300 K	19.2	5.9	27.0	3.4	24.2	1.2	27.9	0.3

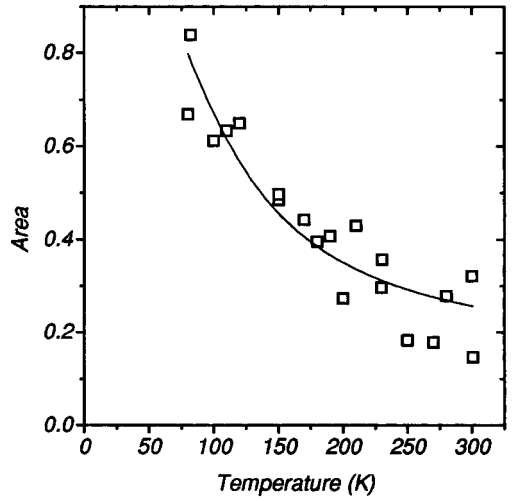

FIGURE 7 Temperature dependence of the 743-nm absorption sub-band intensity for photosystem I trimers of *Spirulina*. The solid line was calculated by assuming seven thermally populated and degenerate (excited) states with a single energy gap of 185 cm^{-1} with the ground state. For details, see text.

ciated with the glass/liquid phase change that occurs at ~170 K for this glycerol concentration.

DISCUSSION

In the present paper a red-absorbing spectral form in *Spirulina* trimers, which absorbs near 709 nm, has been clearly identified by absorption difference and CD spectroscopies. This form is irreversibly bleached by illumination of samples with high-intensity white light. The thermal broadening characteristics of this photobleaching hole, investigated between 80 and 300 K and analyzed in the linear electron-phonon hypothesis, yield a value for the optical reorganization energy ($S\nu_m$) of 52 cm^{-1} and an $FWHM_{inh}$ of 150 cm^{-1}. This value for $S\nu_m$ was confirmed by Gaussian decomposition of the red absorption tail in the same temperature range. Thus, if we assume a mean phonon frequency of ~20 cm^{-1}, as has been invariably observed in chlorophyll/protein complexes by hole burning (e.g., Hayes et al., 1988; Tang et al., 1990) and recently confirmed for a *Synechocystis* sp. red form (Rätsep et al. 2000), the electron-phonon coupling strength for this chlorophyll is $S \approx 2.5$. For normal non-red-shifted chlorophylls S values of 0.5–1 are usually reported (Hayes et al., 1988; Tang et al., 1990), and this is indirectly confirmed here for the bulk antenna of trimers, which have an average reorganization energy of ~14 cm^{-1}. We therefore conclude that the $Q_y(0, 0)$ of the 709-nm chlorophyll form is much more strongly coupled to the low-frequency protein phonons than non-red-shifted antenna Chls. This conclusion, for a specfic Chl form, is in general agreement with our earlier, somewhat more indirect, moments analysis for the entire red absorption tail of PSI200 and LHCI (Croce et al., 1998). The Q_y bandwidth (FWHM) at RT for this form is close to 18 nm and is dominated by the homogeneous (thermal) component (Eq. 3). The Stokes' shift, which is equal to $\sim 2S\nu_m$, is thus expected to be close to 5 nm. It should be noted that although this value for the Stokes' shift is considerably greater than that for normal antenna chlorophylls it is much less than the values often suggested previously for red spectral forms, which fall in the 10–25-nm range (see Introduction). This point requires several comments. The Stokes' shift suggested here for the 709-nm Chl form is based on determination of the optical reorganization energy ($S\nu_m$) by means of an absorption analysis. This Stokes' shift thus has the meaning of the absorption/fluorescence energy separation due to relaxation within the Q_y excited-state manifold of a purely homogeneously broadened pigment band. This is quite different from the apparent Stokes' shift parameter normally reported in absorption/fluorescence experiments as the energy separation between absorption and fluorescence bands; in these cases the Stokes' shift is also determined by the site inhomogeneous energy distribution. If this were large it would substantially increase the apparent Stokes' shift. It should also be borne in mind that determination of the Stokes' shifts on the basis of a comparison of absorption/fluorescence bands in spectrally congested samples (strong overlap between spectral bands) may often be seriously questioned due to excited-state equilibration between spectroscopically unresolved absorption states. For example, a low-energy absorption state, even when present at levels that are experimentally difficult to determine, may be strongly populated at cryogenic temperatures and hence lead to the determination of an erroneously large apparent Stokes' shift. This is apparently the case in *Synechocystis* sp. where the origin band for the 722-nm emission was considered to be derived from the major red form at 708 nm (Gobets et al. 1994), when in fact it has recently been shown to be derived from a very weak absorption state at 714 nm (Rätsep et al., 2000). It is also possible that there is considerable heterogeneity of electron-phonon coupling among the red Chl forms. This may be indicated by the 160-cm^{-1} energy separation between the zero phonon line and a broad and intense phonon wing recently reported by Gobets et al. (1998) for a very low-energy state in site-selected fluorescence measurements, although even in this case some caution in interpretation should be exercised due to the possible presence of very weak, low-energy absorption states. This value (160 cm^{-1}) is similar to that reported by Rätsep et al. (2000) for the 714-nm state in *Synechocystis* sp., however in this case the Stokes' shift was attributed to electron-phonon coupling to both low-frequency (16 cm^{-1}) and high-frequency (~100 cm^{-1}) modes. In the present case of the major 709-nm transition in *Spirulina* we are unable to detect evidence for significantly strong coupling to high-frequency modes, as this would lead to the development of marked band asym-

metry on lowering the temperature (unpublished calculations), which we do not observe.

It has been suggested (Gobets et al., 1998) that strong electron-phonon coupling for red spectral forms could be due to excitonic coupling in Chl dimers or higher aggregates by analogy with the well documented situation for the primary donor special pair in bacterial and plant photosystems (Hayes et al., 1988), and recently Rätsep et al. (2000) have presented Stark spectroscopy and high-pressure experiments supporting this idea for very low temperatures. This suggestion is also in line with the observation presented here that the 709-nm Chl form gives rise to a strong CD signal, although other interpretations are possible. Intense CD signals associated with red forms would seem to be of quite widespread occurrence, as has been previously demonstrated in the green alga *Ostreobium* (Koehne et al., 1999), in *Spirulina* (Shubin and Karapetyan, 1986; Shubin et al., 1993), and in PSI-200 and LHCI from higher plant PSI (unpublished data). In the present case we note that photobleaching induces a decrease in the CD signal near 670 nm, which in normalized spectra, is similar in intensity and opposite in sign to the 709-nm change (Fig. 2) at RT. It is therefore tempting to interpret this observation in terms of a Chl dimer with excitonic bands near 670 and 709 nm, although additional information is required to establish this point with certainty.

Based on the demonstrated presence of the 709-nm Chl form we have attempted to develop a sub-band description of the red absorption tail of *Spirulina* trimers by globally decomposing the red tail, measured at RT, at different times of photobleaching and subsequently propagating this description as a function of temperature down to 80 K. This yielded a minimal sub-band description with band maxima near 709, 720, 733, and 743 nm. The latter band is extremely weak at high temperatures. We note that this wavelength distribution of the red forms is rather similar to that which was recently proposed, on the basis of CD spectroscopy, for the green alga *Ostreobium* (Koehne et al., 1999). On the other hand this description is significantly different from that suggested previously by Karapetyan et al. (1997) for 77 K absorption spectra of isolated *Spirulina* trimers and Koehne and Trissl (1998) for intact *Spirulina* cells at RT. In both these earlier descriptions the major red-absorbing form (709 nm) was absent, probably due to the use of Triton X-100, whereas our data clearly demonstrate its presence and furthermore describe its band shape characteristics, information that subsequently assisted in decomposition of the red tail. There is, however, some agreement with Karapetyan et al. (1997) on the positioning of the lowest energy state at 743 nm. These authors placed it at 746 nm. In the study by Koehne and Trissl (1998) it was near 738.5 nm, which we think may represent a combination of the 733- and 743-nm sub-bands of the present description.

Previous studies on the lowest energy state (743-nm Chl form) in isolated *Spirulina* trimers did not clearly define whether or not it was present at RT, and the possibility was suggested that it may form only at cryogenic temperatures (Karapetyan et al., 1997). We have carefully examined this by both absorption and fluorescence techniques and conclude that it is in fact present at RT, although the oscillator strength is weak, increasing three- to fourfold as the temperature is lowered to 80 K. This increase as the temperature is lowered is smooth, thus suggesting that it is not due to protein conformational changes associated, for example, with solvent phase changes. This marked increase in intensity of an absorption transition with decreasing temperature is unusual but not unique. For example, Srajer and Champion (1991) demonstrated that the 760-nm transition in myoglobin doubles in intensity at cryogenic temperatures and this was attributed to the presence of thermally populated low-lying states that do not contribute to the oscillator strength. In this hypothesis the oscillator strength scales with the ground state population. To understand whether this hypothesis is compatible with the present data we have analyzed the temperature dependence of the 743-nm state using a two-level system and calculating the fractional electronic population on the lower energy level. This has been done using the Boltzmann distribution $\bar{n}_0 = (1/1 + g \times e^{-(hc\Delta\nu/k_BT)})$, where $\bar{n}_0$ is the mean number of particles in the ground state, $\Delta\nu = \nu_1 - \nu_0$ is the energy (cm^{-1}) gap between the two states, g is the degeneracy of the upper energy state, and the other symbols are standard. Fig. 7 shows both the temperature dependence of the 743-nm state in the normalization assumption that two Chl molecules contribute to this transition and the fit in terms of $\bar{n}_0$ giving a single energy gap $\Delta\nu = 185\ cm^{-1}$ and $g = 7$ for the thermally populated state. We wish to emphasize that this fit is not presented to extract exact physical values, particularly as they depend on the population normalization used, but to demonstrate that a general idea of this kind may provide the basis for understanding this unusual temperature dependency.

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