

Triplet and Fluorescing States of the CP47 Antenna Complex of Photosystem II Studied as a Function of Temperature

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ABSTRACT Fluorescence emission and triplet-minus-singlet (T-S) absorption difference spectra of the CP47 core antenna complex of photosystem II were measured as a function of temperature and compared to those of chlorophyll *a* in Triton X-100. Two spectral species were found in the chlorophyll T-S spectra of CP47, which may arise from a difference in ligation of the pigments or from an additional hydrogen bond, similar to what has been found for Chl molecules in a variety of solvents. The T-S spectra show that the lowest lying state in CP47 is at ~ 685 nm and gives rise to fluorescence at 690 nm at 4 K. The fluorescence quantum yield is 0.11 ± 0.03 at 4 K, the chlorophyll triplet yield is 0.16 ± 0.03 . Carotenoid triplets are formed efficiently at 4 K through triplet transfer from chlorophyll with a yield of 0.15 ± 0.02 . The major decay channel of the lowest excited state in CP47 is internal conversion, with a quantum yield of about 0.58. Increase of the temperature results in a broadening and blue shift of the spectra due to the equilibration of the excitation over the antenna pigments. Upon increasing the temperature, a decrease of the fluorescence and triplet yields is observed to, at 270 K, a value of about 55% of the low temperature value. This decrease is significantly larger than of chlorophyll *a* in Triton X-100. Although the coupling to low-frequency phonon or vibration modes of the pigments is probably intermediate in CP47, the temperature dependence of the triplet and fluorescence quantum yield can be modeled using the energy gap law in the strong coupling limit of Englman and Jortner (1970. *J. Mol. Phys.* 18:145–164) for non-radiative decays. This yields for CP47 an average frequency of the promoting/accepting modes of 350 cm^{-1} with an activation energy of 650 cm^{-1} for internal conversion and activationless intersystem crossing to the triplet state through a promoting mode with a frequency of 180 cm^{-1} . For chlorophyll *a* in Triton X-100 the average frequency of the promoting modes for non-radiative decay is very similar, but the activation energy (300 cm^{-1}) is significantly smaller.

INTRODUCTION

The core and peripheral antenna complexes in plants and bacteria serve to harvest light and to transfer excitation energy to the photochemical reaction centers (RCs). Energy transfer and subsequent trapping of the excitation energy in a charge-separated state of the RC are usually very efficient because they are generally much faster than the loss processes in the antenna (for a review van Grondelle et al., 1994).

In the isolated reaction center of photosystem II of green plants, a dynamic, temperature-dependent equilibrium exists between the radical pair state $P_{680}^+ \text{Pheo}^-$ and the excited state of the about 6 Chl and 2 Pheo molecules that constitute the RC. Temperature-dependent fluorescence and triplet yield measurements have shown (Groot et al., 1994) that at $T > 75$ K the dynamic equilibrium shifts to the side of the excited state. The intrinsic excited state decay properties of antenna pigments in the RC and their temperature dependence therefore play an important role in the kinetics of the RC. These properties also determine the observed decrease

of the fluorescence yield at $T > 180$ K in the RC (Booth et al., 1990, Groot et al., 1994).

The three intrinsic decay channels for the excited state of a pigment are radiative and internal conversion directly to the ground state and intersystem crossing to the metastable triplet state (see Avouris et al., 1977, for a review on non-radiative decay processes). The protein framework may influence the conversion of excitation energy into vibrational/phonon energy or heat. In aggregates of pigments, the rate of internal conversion is generally enhanced in comparison with the monomeric species (Alden et al., 1992). The non-radiative decay is temperature dependent because the coupling of the pigment to vibrational or phonon modes of the pigment-protein complex depends on temperature (Englman and Jortner, 1970).

The aim of this study is to obtain a better understanding of the decay processes of the excited state of chlorophyll molecules in a protein matrix and to establish the major excitation decay paths in the light harvesting antenna of green plants. To this end we have accurately measured fluorescence spectra and laser-induced triplet-singlet absorption difference spectra to characterize the various decay channels as a function of temperature down to 4 K in a relatively simple complex containing Chl *a*, i.e., the core antenna protein of photosystem II known as CP47. The CP47 complex is a small, monomeric protein containing 13–15 Chl *a* and 2 β -carotene molecules but no Chl *b* (Barbato et al., 1991, Kwa et al., 1992b). It is characterized by an absorption spectrum that overlaps with the RC absorption spectrum, with a maximum in the Q_y region at 675 nm (van Dorssen et al., 1987;

Received for publication 23 May 1994 and in final form 29 September 1994.

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Abbreviations used: Car, carotene; Chl, chlorophyll; fwhm, full width at half maximum; PS, photosystem; T-S, triplet-minus-singlet.

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0006-3495/95/01/281/10 \$2.00

Ghanotakis et al. 1989; van Kan et al., 1992). It is believed to give rise to the red-most fluorescence at 695 nm in the PS II core complex (van Dorssen et al., 1987). A number of observations have suggested that the core antenna proteins have, in addition to the obvious light-harvesting functions, some structural functions as well. The CP47 protein may provide a binding site for the Mn-stabilizing 33-kDa protein (Bricker et al., 1988), and may facilitate the light-induced formation of a tyrosine radical in the reaction center (Petersen et al., 1990).

In this contribution we determine the absolute quantum yields of radiative decay and intersystem crossing of CP47 as a function of temperature, which allows us to model the temperature-dependent decay processes. All measurements are compared with those on "free" chlorophyll *a* in detergent (Triton X-100). In another paper (Groot et al., 1994) this information has been used to model the T-dependence of the yield of fluorescence and triplet formation in a more complicated system such as the isolated D₁D₂-cyt b559 complex.

MATERIALS AND METHODS

CP47 complexes were isolated from spinach as described before (Dekker et al., 1989, 1990; van Kan et al., 1992). For all except the temperature-dependent fluorescence emission experiments, the particles were repurified to remove any free pigment. After desalting on a PD10 column, the CP47 complexes were loaded on a Q-Sepharose anion exchange column for a second time and eluted by a buffer with higher (35 instead of 20 mM) MgSO₄-concentration to obtain a high concentration. Since uncoupled Chl emits at 674.5 nm (see Results and Kwa et al., 1994b) it could easily be observed from the 4 K emission spectra that this procedure removed all chlorophylls disconnected from the protein. Free Chl *a* was obtained from a treatment of CP47 samples with Triton X-100 (Kwa et al., 1994b). It was shown (Kwa et al., 1994b) that this procedure destroys the protein structure and generates monomeric Chl *a* in detergent.

The samples were diluted in a buffer containing 20 mM BisTris, pH 6.5, 20 mM NaCl, 0.03% (w/v) dodecyl maltoside and 80% (v/v) glycerol. The samples for the fluorescence experiments were diluted to an OD of ≤ 0.1 cm⁻¹ at 675 nm, while for the absorption difference experiments an OD of 0.5–0.7 cm⁻¹ was used. The measurements were performed in an acrylic cuvette with a path length of 1 cm. An Oxford Instruments CF 1204 (Oxford, UK) helium flow cryostat in conjunction with an Oxford ITC 4 temperature controller was used to vary the temperature. Absorption spectra were measured on a Cary219 spectrophotometer.

Emission spectra were recorded on a home-built fluorimeter described and corrected for the sensitivity of the system as in (Kwa et al., 1992b). The excitation source was a 150 W tungsten halogen lamp equipped with a 1/8 m monochromator set at 610 nm with a spectral window of 12 nm. This particular wavelength ensures nonselective excitation of the pigments and temperature-independence of the number of absorbed photons. The spectra were measured with a resolution of 1.0 nm.

Laser-flash induced triplet-minus-singlet absorption difference spectra were measured on a home-built single-beam spectrophotometer. The triplet states were induced by a dye laser (PDL2, Quanta-Ray) pumped by a frequency doubled Nd:YAG laser (DCRA, Quanta-Ray) operating at 1 Hz. For excitation at 610 nm the dye sulforhodamine 101 was used, pulses of 8 ns (fwhm) at an intensity of about 10–15 mJ were produced. For excitation between 680 and 720 nm, the dye pyridine was used (output intensity ~ 4 mJ). The laser intensity was measured before and after the absorption difference spectra were recorded with a Scientech 38-0101 energy monitor. To ensure a homogeneous distribution of excitation light a diffuser was placed on the entrance window of the cryostat. The probe light source was a 150 W tungsten halogen lamp. In the region between 650 and 750 nm the transmitted light was detected with a Centric OSD 60-3T photodiode with a

low-noise preamplifier, with a response time of 8 ms. An RG 610 filter and a shutter were used to reduce the actinic effect of the probe lamp. T-S spectra between 400 and 600 nm were measured with a Hamamatsu R928 photomultiplier, with a response time of 3 μ s, and a GG 420 filter in front of the sample. The detectors were placed behind a 1/4 monochromator (PTI). The traces were recorded at a resolution of 1 nm, digitized by a Lecroy 9310 transient recorder and stored on computer. Decay-associated spectra were estimated from a multi-response analysis (Knutson et al., 1983; van Stokkum et al., 1994) of kinetic traces recorded at 30–50 probe wavelengths.

Triplet yields were determined from the slope at zero intensity of saturation curves of the Δ OD signal recorded at the wavelength of maximal bleaching. The quantum yield of two separate components was obtained from a multi-response analysis of the saturation curves. The fraction of chlorophyll molecules converted into the triplet state was approximated by the ratio of the integrals over the T-S and absorption spectra from 650 nm to 700 nm. The small contribution of triplet-triplet absorption is not taken into account, since the relative contribution will be approximately equal in free Chl and CP47. For the CP47 complex the relative number of triplets per pigment is converted to the number of triplets per CP47, by multiplying by 14, the most probable number of pigments in the protein (Barbato et al., 1991; Kwa et al., 1992b). The exact number of photons at a certain laser intensity incident on the sample was calibrated with free Chl *a* at 77 K for which a triplet quantum yield of 0.64 was assumed (Seely et al., 1986). The β -carotene triplet yield was calculated using a triplet extinction coefficient of 2.4×10^5 M⁻¹cm⁻¹ at 530 nm (Bensasson et al., 1983). The absolute fluorescence quantum yield (Φ_F) was obtained from a comparison of the integral of the emission spectrum from 650 nm to 750 nm with that of free Chl *a* in Triton X-100 at 77 K, for which a quantum yield of 0.30 was assumed (Seely et al., 1986). In the light of the observed temperature dependence of the Chl fluorescence yield (see Results), the Φ_F of Chl in Triton X-100 at 77 K was compared to Φ_F of Chl in methanol at room temperature. With Φ_F (methanol, RT) = 0.22 (Seely et al., 1986), a yield of Φ_F (Triton X-100, 77 K) = 0.29 ± 0.03 was found.

RESULTS

Absorption spectra

Fig. 1 shows the absorption spectra of CP47 at 4 K (*solid line*) and free Chl *a* (Triton X-100) at 77 K (*dashed line*) in the Q_y region. The spectrum of free Chl *a* is characterized by a single transition at 670 nm and a fwhm of 16.5 nm (Kwa et al., 1994b). The spectrum of CP47 is characterized by a set of distinct absorption bands, probably reflecting Chl *a* molecules in different environments within the CP47 complex. The maxima in the second derivative are at 661, 669.5, 676, and 682.5 nm. The spectrum shows more structure than the spectrum of another preparation reported by van Dorssen et al. (1987). The 4 K spectrum also shows somewhat more fine structure than the 77 K spectrum (van Kan et al., 1992). The minor contribution at 691 nm observed by van Dorssen in the 4th derivative is not present in the 2nd derivative of our spectrum. However, also in our case the absorption extends to ~ 700 nm, which suggests that such a contribution is also present in our preparation. The inset of Fig. 1 shows the absorption spectrum of CP47 from 350 to 750 nm, in which the absorption of the β -carotenes at 467 and 502 nm is clearly visible.

Fluorescence spectra

To gain insight in the distribution of energy levels and the localization of the excited state in the CP47 antenna, we

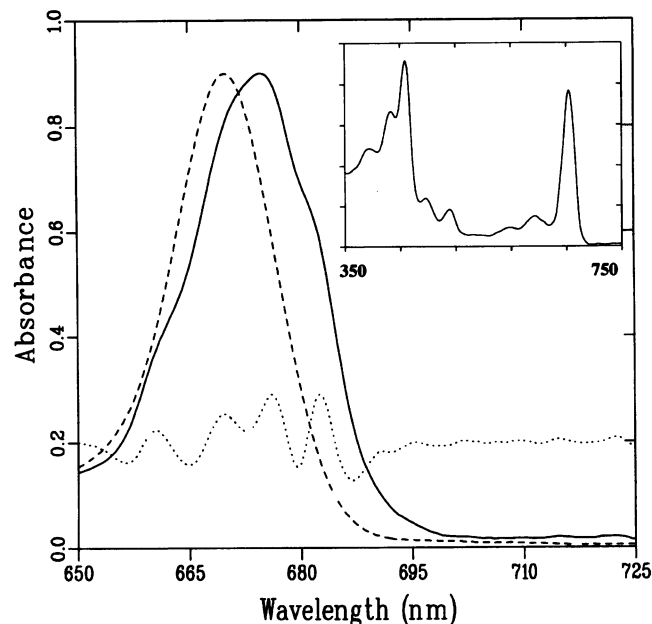


FIGURE 1 Q_y region of the absorption spectrum of the CP47 complex at 4 K (solid line) and its second derivative (multiplied by -5 , dotted line) and of Chl a at 77 K (Triton, dashed line). The inset shows the CP47 absorption spectrum from 750–350 nm.

recorded fluorescence emission spectra as a function of temperature and compared the quantum yields with those of free Chl in Triton X-100.

Fig. 2 shows the emission spectra of repurified CP47 and monomeric Chl at 4 K, excited at 610 nm. The Chl spectrum (dashed line) peaks at 674.5 ± 1.0 nm and has a fwhm of 17 nm. The CP47 emission (solid line) is characterized by

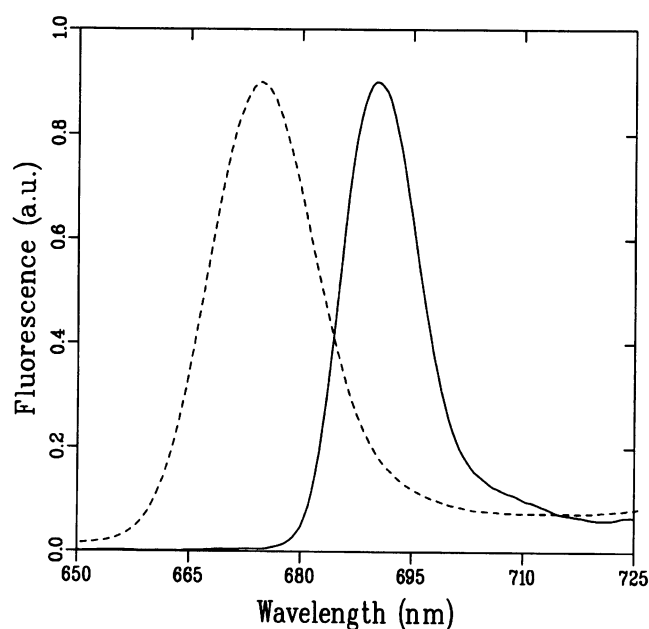


FIGURE 2 Emission spectra of CP47 (solid line) and Chl a (Triton, dashed line) at 4 K upon excitation at 610 nm.

a maximum at 690 ± 1.0 nm and a fwhm of 12.5 nm. The spectrum and the second derivative (not shown) do not show any distinct shoulders or multiple maxima. These characteristics differ somewhat from those observed by van Dorssen et al. (1987), peak wavelength 693 nm and fwhm 15 nm.

At 4 K the absolute fluorescence quantum yield of the CP47 complex is 0.11 ± 0.03 , which is about 3 times lower than the fluorescence yield of free Chl a .

Temperature effect

Fig. 3 shows the emission spectra of CP47 between 4 K and 270 K. For these experiments preparations were used that had not been repurified. The spectra show a minor emission band at 675 nm, with an amplitude of 5% of the maximum at 690 nm at 4 K, due to a small amount of uncoupled Chl a . With the CP47 fluorescence yield of 0.11 at 4 K, the fluorescence yield of free Chl (0.30) and the pigment concentration of 14, it can be estimated that there is one uncoupled Chl pigment on every four CP47 complexes.

Upon raising the temperature, the maximum of the emission band gradually shifts from 690 nm at 80 K to 683 nm at 190 K. Below 80 K and above 190 K almost no shift is observed. The emission band broadens from a fwhm of 12.5 nm at 4 K to 18 nm at 270 K. The spectral shift and broadening is explained by thermal equilibration of excitations over the different spectral forms.

The spectrum of free Chl does not depend very much on the temperature; the peak position does not change, while the width increases from a fwhm of 17 nm at 4 K to 21.5 nm at 270 K (not shown).

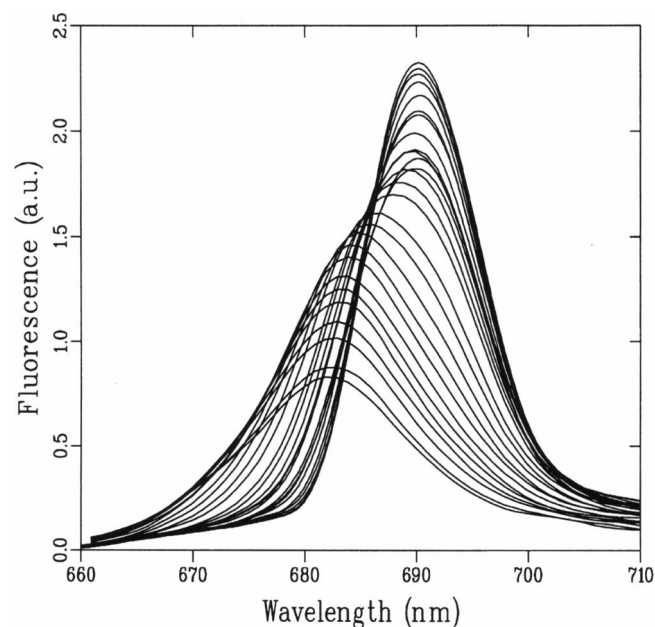


FIGURE 3 Fluorescence emission spectra of the CP47 complex recorded at 26 temperatures between 4 and 270 K. The emission maximum at 4 K is at 690 ± 1.0 nm, fwhm 12.5 nm, above 70 K the spectra shift and broaden to 683 nm and 18.0 nm at 270 K.

When the integrated areas of the Chl *a* and CP47 emission spectra are plotted, a moderate temperature effect is observed for both samples (Fig. 4); the fluorescence quantum yields are constant at low temperature and start to decrease above 120 K. The relative decrease is stronger for CP47 than for free Chl *a*: at 270 K the fluorescence quantum yield of CP47 is decreased to 55% of its low temperature value, while the yield of free Chl *a* is decreased to about 80% of its low temperature value. The uncertainty in the value of the quantum yields relative to each other is negligible (we estimate the experimental uncertainty due to systematic errors to be 0.03).

Characterization of triplet states

The decay channel of the excited state to the metastable triplet state via intersystem crossing was probed by light-minus-dark absorption difference spectroscopy. After excitation with a short laser pulse the Chl *a* and Car triplet states were probed in the millisecond and microsecond time ranges, respectively. Additional information on the decay components that contribute to the spectra was obtained by a kinetic decomposition (Knutson et al., 1983; van Stokkum et al., 1994). The triplet-minus-singlet difference spectra are expected to contain the spectra of the pigment(s) which are converted to the triplet state and those of neighboring pigments, the absorption properties of which have changed by the formation of a triplet state in the pigment-protein complex (van Mourik et al., 1992).

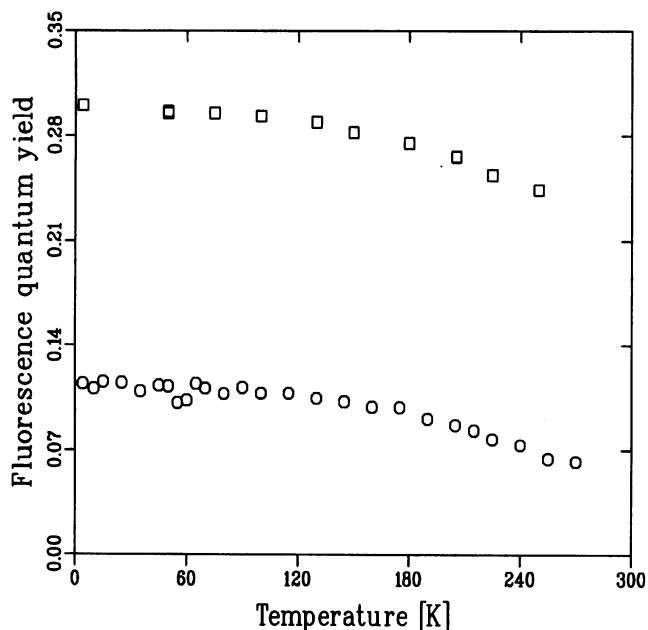


FIGURE 4 Fluorescence quantum yield of CP47 (circles) and Chl *a* (Triton, squares) as a function of temperature. The CP47 fluorescence yield decreases from 0.11 at 4 K to 0.06 at 270 K, the Chl *a* (Triton) fluorescence yield from 0.30 at 4 K to 0.25 at 250 K.

Chlorophyll triplet-minus-singlet spectra

Fig. 5 shows the result of a multi-response analysis of the decay kinetics of Chl *a* (Triton X-100) at 77 K. A best fit is found using two components with different decay rates and spectra. The decay-associated spectra have minima at 668 ± 1 nm and 673 ± 1 nm (Fig. 5 *a*) and lifetimes of 2.8 ms and 1.6 ms, respectively (Fig. 5 *b*). The combined spectrum at $t = 0$ could be fitted very well with the ground state absorption spectrum and a triplet-triplet absorption spectrum that is constant with wavelength, with an amplitude of 10% of the maximal bleaching (not shown).

The multi-response analysis of the CP47 data at 4 K is shown in Fig. 5, *c* and *d*. The ground state absorption recovery is biphasic, with lifetimes of 2.0 and 0.6 ms. The decay-associated spectra have their maximum bleaching at 684 ± 1 nm and 686 ± 1 nm, respectively. Both spectra are characterized by a fwhm of the main band of 8 nm and in particular the 686-nm spectrum shows a small additional minimum around 670 nm which cannot be due to uncoupled Chl. The T-S spectra coincide with the observations of Jankowiak and Small (1993) who ascribed their results to a state in the CP47 complex absorbing at ~ 686 nm. The triplet quantum yields are 0.05 ± 0.02 and 0.11 ± 0.02 , for the 684 and 686 nm pigments, respectively.

Carotenoid triplet-minus-singlet spectra

In the CP47 complex also β -carotene triplet states are formed upon illumination. These triplet states are not formed directly through the decay of the excited Car state (because β -carotene does not absorb at the 610-nm excitation wavelength) but through triplet energy transfer from a Chl^T state. Fig. 6 shows the multi-response analysis of T-S data recorded at 40 K. The structured spectrum with maxima at 490 and 530 nm and minima at 470 and 505 nm has a lifetime of 11 μ s. The spectrum is very similar to the β -carotene T-S spectrum in CP47 reported by Carbonera et al. (1992), the triplet lifetime is close to that of β -carotene in hexane (Bensasson et al., 1983). The second component does not decay on the time scale of the experiment. It has a maximum around 470 nm and is characteristic for Chl triplet states. The spectrum recorded at $T = 4$ K could be fitted including a biexponential decay for the carotene-triplet difference spectrum; $\tau_1 = 10$ μ s, $\tau_2 = 43$ μ s (not shown). At low temperature, triplet sub-levels are not in equilibrium, due to which the two components may reflect the decay from two of the individual sub-levels. The quantum yield of Car triplet formation was determined to be 0.15 ± 0.02 at 4 K.

Temperature effect

The kinetically unresolved T-S spectra in the Chl Q_y region measured at 4, 40, 110, 160, and 200 K are shown in Fig. 7. The main bleaching shifts from 685 nm at 40 K to 681.5 nm at 200 K and broadens from 9 nm at 40 K to 13 nm at 200

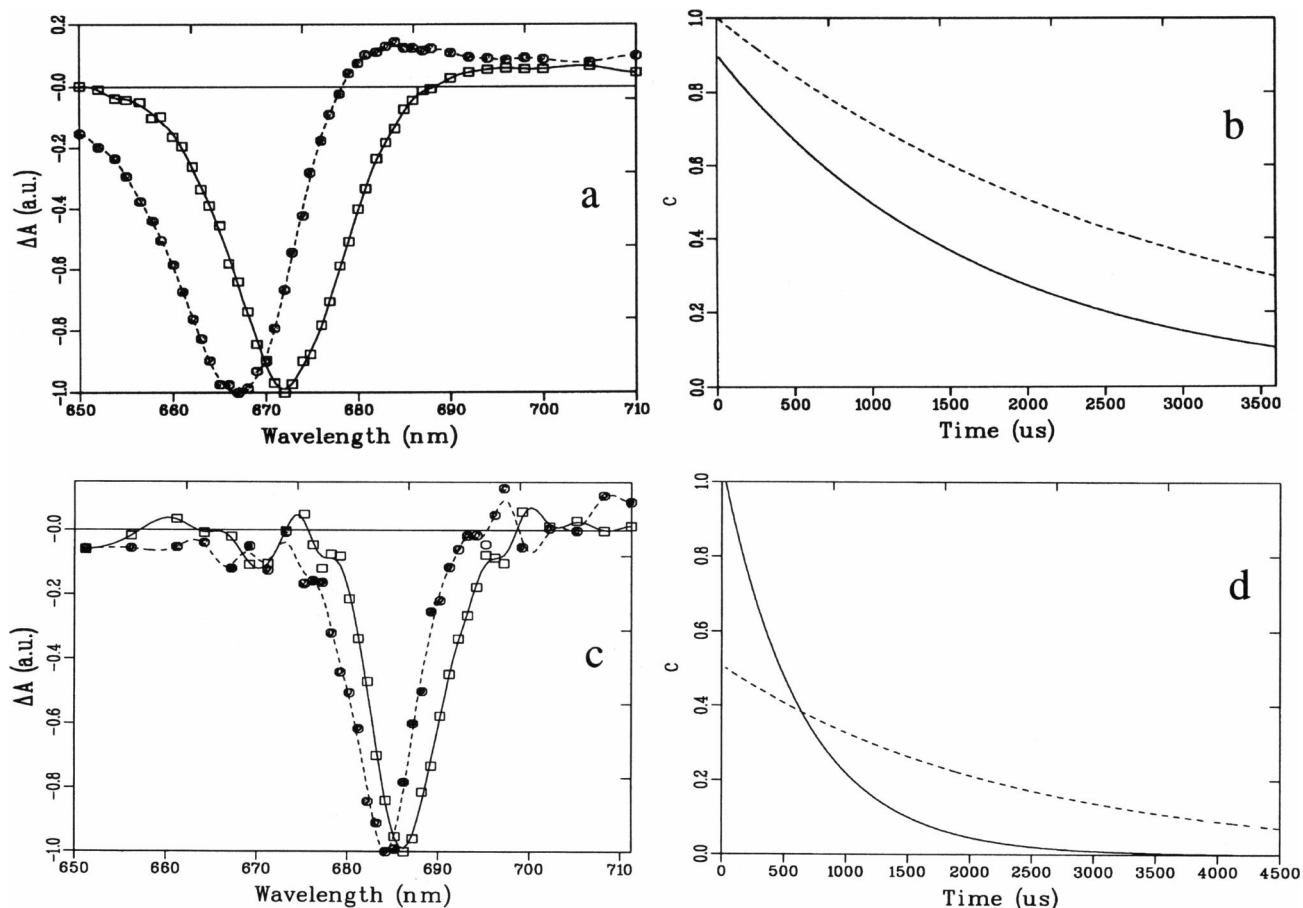


FIGURE 5 Triplet-singlet absorption difference spectrum of Chl *a* (Triton) at 77 K; multi-response analysis of time traces recorded at 43 wavelengths, decay-associated spectrum (a) and kinetic vectors (b). A best fit is found using two components; one with maximal bleaching at 668 ± 1 nm and a triplet lifetime of 1.6 ms and the other at 673 ± 1 with a lifetime of 2.8 ms. T-S spectrum of the CP47 complex at 4 K; multiresponse analysis of kinetic traces recorded at 36 wavelengths, decay-associated spectrum (c) and kinetic vectors (d). A best fit is found using two components: the spectrum with maximal bleaching at 684 ± 1 nm has a triplet lifetime of 2.0 ms, the other with the maximal bleaching at 686 ± 1 nm has a triplet lifetime of 0.6 ms.

K, which is presumably largely due to the equilibration of excitation energy over all pigments. The temperature dependence of the total Chl^T quantum yield (Fig. 8, squares) is different from that of the fluorescence yield. At 4 K the yield is 0.16 ± 0.03 , but it decreases almost linearly to 0.07 at 185 K. The carotenoid T-S spectrum is almost independent of temperature (a minor broadening is observed; data not shown); the carotenoid triplet yield is independent of temperature between 4 and ~ 135 K, while it decreases to 0.12 ± 0.02 at 185 K (Fig. 8, circles).

Site-selected carotenoid and chlorophyll triplet yield

In Table 1, the fwhm and maxima of the emission and T-S spectra of CP47 at several temperatures are given. Both the T-S spectra and the emission spectra are believed to originate from the lowest energy state(s) in CP47. Consequently the T-S and emission spectra are expected to exhibit similar temperature effects. However, the emission maximum shifts more to the red upon cooling to 4 K than the T-S spectrum,

and the T-S spectra are always about 4 nm narrower than the emission spectra. One possible reason for this discrepancy could be that there is a special path for the triplet-triplet transfer from Chl^T to Car^T , involving mainly “red” Chl *a*'s of CP47. To decide whether or not such a special path exists in CP47 the following experiment was performed. The yield of Chl^T and Car^T formation was monitored upon excitation at the very red edge of the absorption band at 685 and 690 nm. If there would be a population of red pigments that would transfer its triplet state directly to a β -carotene (and thus remain undetected in the Chl T-S spectrum), there would be a relative increase of the Car^T yield upon excitation at 690 nm.

The Car triplet yield at 4 K was detected at the maximum absorption increase at 530 nm and at 540 nm as a function of the excitation wavelength (the T-S spectrum upon excitation at 685 nm was identical to the spectrum induced upon 610 nm excitation, not shown). The Chl triplet yield was constructed from the maximal amplitude of bleaching, which coincided with the excitation wavelength, as a function of excitation wavelength.

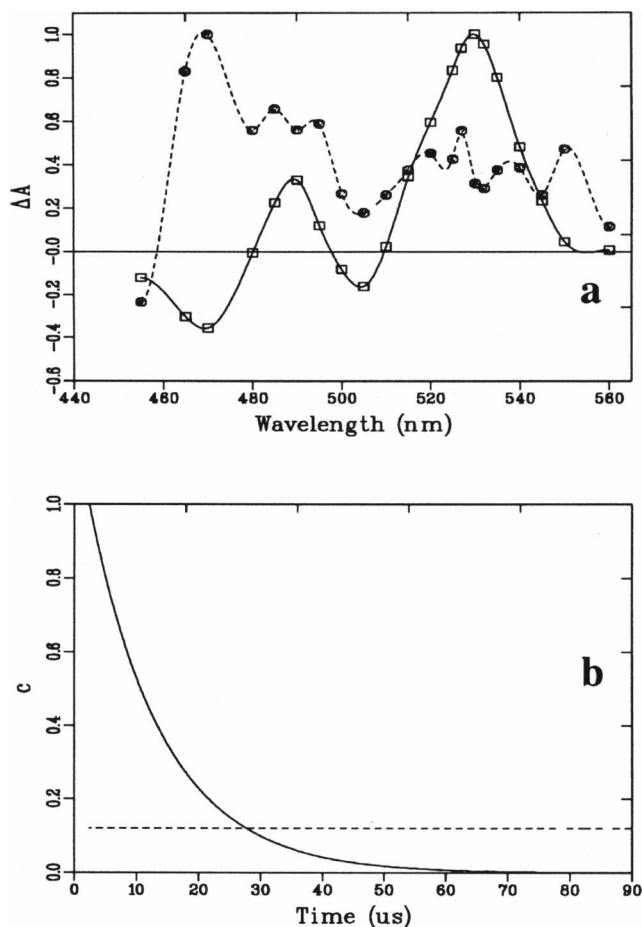


FIGURE 6 Flash-induced T-S spectrum of CP47 at 40 K in the carotenoid region upon 610 nm excitation recorded at 22 wavelengths, decay-associated spectrum (a) and decay vectors (b). The decay to the ground state is composed of a component with a decay rate of $(11 \mu\text{s})^{-1}$ and a spectrum with extrema at 470, 490, 505, and 530 nm. The second component does not decay on the time scale of the experiment ($90 \mu\text{s}$) and has a decay-associated spectrum with a maximum at 470 nm.

The ratio of Car and Chl triplet yields is 0.9 ± 0.1 upon 690 nm excitation. This ratio is about the same as the ratio obtained at 685 nm excitation (1.1 ± 0.1). We conclude therefore that there is no evidence for a red pigment pool that transfers its triplet state preferentially to the carotenoids.

DISCUSSION

Spectral changes upon triplet formation

From the temperature dependencies of the emission and T-S spectra of CP47 and free Chl *a* (Triton X-100) (Table 1) the following observations can be made. 1) For CP47, but not for free Chl, the peak maxima of the emission and S-T spectra depend on the temperature. 2) For free Chl, but not for CP47, the width of the T-S spectrum is about the same as the width of the emission spectrum. 3) The Chl T-S spectrum in the CP47 complex is less asymmetric (shows less bleaching on the blue side of the minimum) than the free Chl T-S spectrum. 4) The CP47 T-S spectra, but not those of free Chl,

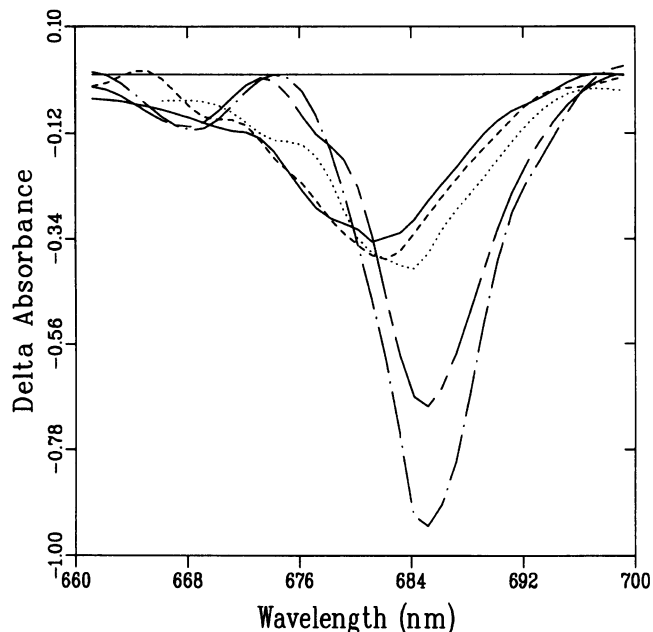


FIGURE 7 Chl T-S spectra in the CP47 complex recorded at 4, 40, 110, 160, and 200 K. The spectra are constructed from the average amplitude of the time traces the first $200 \mu\text{s}$ after the excitation flash. The spectra shift and broaden from $685 \pm 1 \text{ nm}$ and a fwhm of 8.5 nm at 4 K to $681 \pm 1 \text{ nm}$ and a fwhm of 13 nm at 200 K.

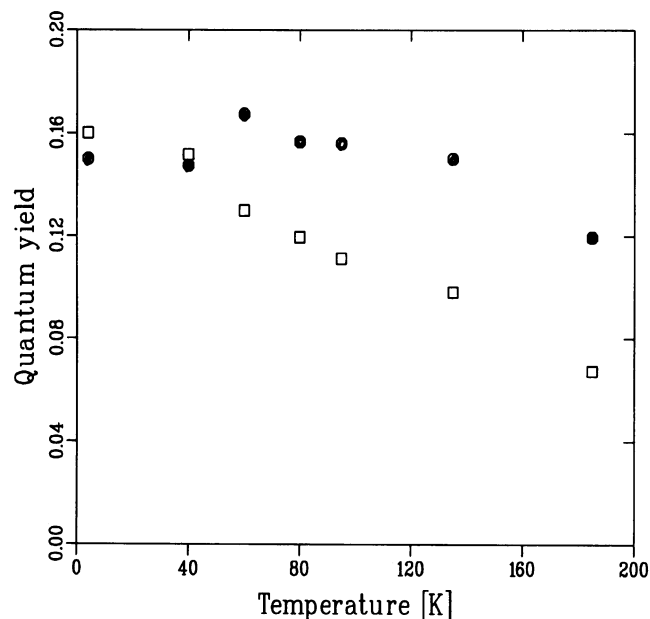


FIGURE 8 The quantum yield of chlorophyll (squares) and β -carotene (circles) triplet formation as a function of temperature.

resemble the T-S spectra of the RC of PS II at 4 K (van Kan et al., 1990; Kwa et al., 1994a; Groot et al., 1994) in the sense that a positive feature on the blue side of the main bleaching and an additional bleaching around 670 nm were observed.

Since the site-selective excited triplet yields in CP47 have shown that there is no special triplet transfer path of "red" Chls to Car pigments, we conclude that the observed dif-

TABLE 1

<i>T</i> [K]	Emission maximum (nm) ± 1.5	T-S maximum (nm) ± 1 nm	fwhm emission (nm) ± 1.5	fwhm T-S (nm) ± 1 nm
	nm	(nm) ± 1 nm	nm	(nm) ± 1 nm
4	690	685	12.5	8.5
40	690	685	13	8.5
110	685	684	17	11.5
160	684	682	17.5	13
200	683	681	17	13

ferences between the emission and T-S spectra of CP47 and free Chl (features 1–4), are a direct effect of the formation of a Chl triplet state in the CP47 complex. The triplet state probably induces changes in the electrostatic interactions with nearby pigments. For example, the localization of a triplet state on a pigment may lead to the breaking of exciton interactions in dimeric or oligomeric structures of pigments (the CP47 complex has a large CD signal; see Kwa et al., 1994b). The absorption of the newly formed state of the remaining set of interacting pigments may partly cancel out the bleaching of the original ground state absorption. The T-S spectrum in the PS II RC is explained in the same manner by Kwa et al. (1994a). The superposition of spectra due to bleaching, absorption recovery, and bandshifts in combination with equilibration over different spectral forms may lead to the observed complex effects on the T-S spectra when the temperature is varied.

Biexponential decay of triplet states

The multi-response analysis of the T-S spectra of both CP47 and Chl *a* (Triton X-100) yielded two spectral components with different triplet state lifetimes, where the triplet lifetime of the component with the lower $S_0 \rightarrow S_1$ transition is shorter. Since the spectra of the two decay times are different, it is not likely that the two decay times arise from the sub-levels of one triplet state (as was observed for the P680 triplet of PS II at $T < 40$ K; Groot et al., 1994). However, this effect may certainly play a role and complicate the interpretation of the two components, especially in the CP47 T-S spectrum that is recorded at a temperature (4 K), where the triplet sublevels are not in equilibrium.

Chlorophyll pigments in several solvents, such as pyridine, *n*-octane and methanol may be mono- or bisligated (Mauring et al., 1987). The triplet lifetime of the mono- (L_1) and bisligated (L_2) pigments differs, which may be accounted for by the fact that the energy difference of the $T_1 \rightarrow S_0$ transition is considerably lower for bisligated pigments (Mauring et al., 1987; Krasnovskii, 1977). Monoligated and bisligated pigments have average triplet lifetimes of 2.5 and 1.4 ms, respectively, while hydrogen bridges (denoted L_1H or L_2H) further shorten the lifetime of bisligated pigments to 0.8 ms (L_1H type pigments have the same lifetime as L_1 pigments; Mauring et al., 1987). The $S_0 \rightarrow S_1$ transition is at somewhat lower energy for the bisligated pigments.

Our experimental results on Chl *a* (Triton X-100) and CP47 point to similar tendencies: shorter triplet lifetimes for

the longer wavelength components. Therefore the two spectral components may originate from a difference in coordination of the central magnesium atom of the chlorophyll pigments in CP47 and Chl *a* (Triton X-100). The 0.6-ms lifetime of the 686-nm component in the CP47 T-S spectrum is very close to the cited average value of 0.8 ms for L_2H solvated pigments, suggesting that at least one pigment in CP47 is six-coordinated and has a hydrogen bond. This assignment is supported by a shoulder in the absorption spectrum at 637 nm near the band at 625 nm, which are ascribed to L_2 and L_1 solvated pigments, respectively (Renge and Avarmaa, 1985). In a recent resonance Raman study of the CP47 complex, de Paula et al. (1994) assigned a vibration at ~ 1670 cm^{-1} to the hydrogen-bonded carbonyl group of part of the chlorophylls. All the chlorophylls were, however, assigned to be five-coordinated, which was based on the observation of two bands at 1563 cm^{-1} and 1617 cm^{-1} . For six-coordinated chlorophyll, these bands would presumably 5 cm^{-1} and 11 cm^{-1} be shifted downward, respectively (Fujiwara and Tasumi, 1986). In our opinion, it is very possible that such a small shift will not be observed (with a spectral resolution of 6–8 cm^{-1}) when a minor amount of the about 14 chlorophylls is six-coordinated.

Temperature dependence of Φ_F and Φ_T

The decrease with temperature of Φ_F of free Chl *a* with 20% is an effect that to our knowledge has not been reported before. With time-resolved fluorescence experiments, Booth et al. (1990) found the yield of the 6-ns component, which they ascribed to Chl uncoupled from charge separation, to decrease with only 2% from 77 K to 300 K. However, this was measured in the isolated D_1 - D_2 cyt b559 complex, where the kinetics of recombination luminescence (of the reaction $P^+I^- \rightarrow P^*$) become faster upon raising the temperature, to reach lifetimes of ~ 6 ns above 150 K. Also the amplitude of lifetimes in this time domain increases with temperature (Roelofs et al., 1993). As a consequence, lifetimes of this origin may have been wrongly ascribed to uncoupled Chl, thereby hindering the observation of the true decrease of Φ_F of uncoupled Chl with temperature.

The decrease of the CP47 fluorescence yield is much larger (about 50% from 120 K to 270 K). A similar decrease was observed for the total triplet yield. Since the carotenoid triplet states are formed via chlorophyll triplet states, the total triplet yield in CP47 is calculated by taking the sum of $\Phi_T(\text{Car})$ and $\Phi_T(\text{Chl})$, i.e., 0.31 ± 0.05 at 4 K. This suggests that the major decay channel for the lowest excited singlet state in CP47 is via internal conversion, because the quantum yield of this process will be $(1 - 0.31 - 0.11) = 0.58$ at 4 K.

We propose that the decrease in Φ_F and Φ_T with increasing temperature is caused by an increase of the non-radiative decay rate with temperature. Englman and Jortner (1970) derived temperature-dependent expressions for non-radiative transitions between two electronic states, both in the strong coupling and the weak coupling limit. The weak coupling limit is encountered when the relative horizontal

displacement of the harmonic potential energy surfaces is small (Fig. 9), or $E_M \sim \hbar\omega_m$, in which E_M is half the Stokes shift and ω_m the mean vibrational frequency of the modes coupled to the non-radiative transition. In the strong coupling limit the relative displacement between the potentials is large and the transition between the two electronic states is characterized by a large Stokes shift, or $E_M \gg \hbar\omega_m$.

We estimate E_M from the T-S and emission spectra to be ~ 50 – 70 cm^{-1} for both CP47 and Chl *a* (Triton X-100). Kwa et al. (1994b) showed by site-selective excitation in the $Q_{y(0-0)}$ region of Chl *a* (Triton X-100) that the width of the emission bands is broad, 5–10 nm or 100–200 cm^{-1} , and mainly determined by phonon side bands. The coupling strength can be calculated (Englman and Jortner, 1970) for several temperatures and mean vibrational frequencies. It is always of the order of the Stokes shift, which is not clearly in the weak-coupling limit. Probably, for both CP47 and Chl *a* (Triton X-100), the coupling of the electronic states to phonon modes is intermediate, neither weak nor strong.

In the following we will nevertheless use the expression for the non-radiative rate in the strong coupling limit to fit the Φ_F and Φ_T curves, mainly because in the weak coupling limit information is required on the number of quanta in each low-frequency mode which is not yet available. In the future we plan to record site-selective emission and T-S spectra from which the distribution of phonon modes may be estimated, similar to what has been done in (Pullerits et al., 1994). Recently, Hwang-Schweitzer (1992) applied the strong coupling limit for similar reasons to non-radiative decay to describe the fluorescence intensity of photosystem II membrane preparations as a function of temperature.

In the strong coupling limit the non-radiative decay rate is given by:

$$k_{nr}(T) = \frac{C}{(k_B T_{eff})^{1/2}} \exp\left(-\frac{E_A}{k_B T_{eff}}\right) \quad (1)$$

where the effective temperature T_{eff} is defined as;

$$k_B T_{eff} = \frac{1}{2} \hbar \omega_m \coth(\beta \hbar \omega_m / 2) \quad (2)$$

where k_{nr} is the sum of internal conversion (i.e., the con-

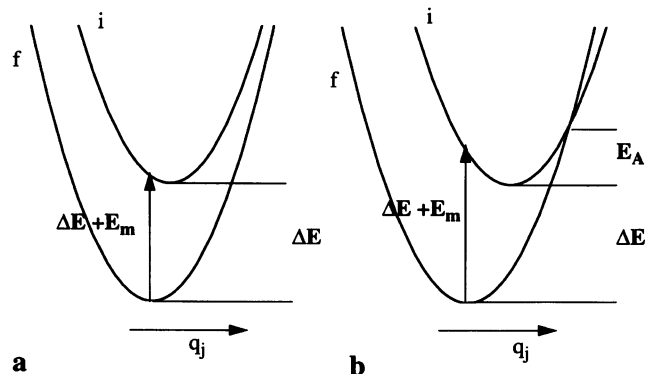


FIGURE 9 Schematic representation of the potential energy surfaces of the initial (i) and final state (f) as a function of the reaction coordinates q_j in the weak (a) and the strong (b) coupling case.

version of excitation energy into vibrational, phonon energy, or heat) and intersystem crossing to the triplet state, $k_{nr} = k_{isc} + k_{ic}$; C is a constant, the sum of the absolute transition rates of internal conversion and intersystem crossing at $T = 0$ K ($k_{ic}(0)$ and $k_{isc}(0)$); $\beta = 1/k_B T$; ω_m the mean vibrational frequency and E_A the activation energy which is measured from the minimum of the initial potential curve to the point where the two potentials intersect.

Φ_F and Φ_T are defined in terms of the transitions rates as:

$$\Phi_F = \frac{k_r}{k_r + k_{isc} + k_{ic}} \quad \text{and} \quad \Phi_T = \frac{k_{isc}}{k_r + k_{isc} + k_{ic}} \quad (3)$$

The radiative decay rate k_r of free chlorophyll can be calculated with the Strickler-Berg relation or estimated from the quantum yield of fluorescence (0.30) and the excited state lifetime (6 ns) (Seely et al., 1986), i.e., $k_r \sim 0.05$ ns^{-1} . For CP47 we assume the same value. From $\Phi_F(4\text{ K}) = 0.11$ and $\Phi_T(4\text{ K}) = 0.31$, we estimate $k_{isc}(0)$ to be $2.7 \times k_r = 0.14$ ns^{-1} and $k_{ic}(0)$ to be $5.1 \times k_r = 0.25$ ns^{-1} for CP47.

For CP47 $k_{nr}(T)$ is found by fitting $\Phi_T(T)$, using eq. 1–3, to the experimental data which yields $E_A(k_{nr})$ and $\omega_m(k_{nr})$. Insertion of the parameters $E_A(k_{nr})$ and $\omega_m(k_{nr})$ in $\Phi_T(T)$ does, however, not produce a good fit to the triplet quantum yield data. Instead, an almost constant curve is calculated. Therefore we have fitted the triplet yield data using different

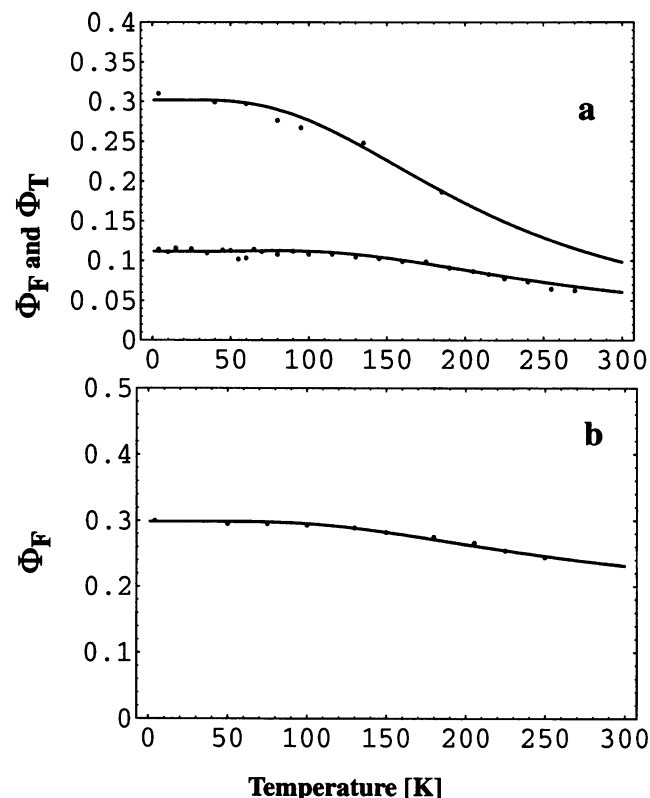


FIGURE 10 (a) Simulation of the CP47 total triplet (upper curve) and fluorescence (lower curve) yield data using Eqs. 1–3 and $E_A(k_{isc}) = -10$ cm^{-1} , $\omega_m(k_{isc}) = 180$ cm^{-1} , $E_A(k_{ic}) = 650$ cm^{-1} and $\omega_m(k_{ic}) = 350$ cm^{-1} . (b) Simulation of the Chl *a* (Triton) fluorescence yield using Eqs. 1–3, $E_A(k_{nr}) = 300$ cm^{-1} and $\omega_m(k_{nr}) = 350$ cm^{-1} .

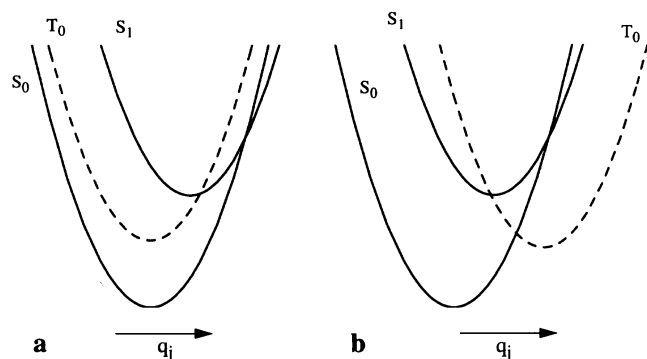


FIGURE 11 Two configuration of the adiabatic potentials of the states S_0 , S_1 , and T_0 , that are suggested by the activationless behavior of the inter-system crossing rate from S_1 to T_0 .

E_A and ω_m values for k_{isc} which we label $E_A(k_{isc})$ and $\omega_m(k_{isc})$, while $k_{nr}(T)$ is used in the denominator. This yields $E_A(k_{isc}) = -10 \text{ cm}^{-1}$ and $\omega_m(k_{isc}) = 180 \text{ cm}^{-1}$ (Fig. 11 a, upper curve). Now the E_A and ω_m values for k_{ic} can be found from $k_{nr}(T)$ and $k_{isc}(T)$. This yields $E_A(k_{ic}) = 650 \text{ cm}^{-1}$ and $\omega_m(k_{ic}) = 350 \text{ cm}^{-1}$ (Fig. 10 a, lower curve). The Chl *a* (Triton X-100) fluorescence yield can be fitted using $E_A(k_{nr}) = 300 \text{ cm}^{-1}$ and $\omega_m(k_{nr}) = 350 \text{ cm}^{-1}$ (Fig. 10 b). The values are determined by the experimental curves within 30 cm^{-1} .

We note that good fits are obtained using a value of about 350 cm^{-1} for the promoting/accepting mode in the internal conversion process, which is a typical molecular vibration frequency of chlorophyll (Avarmaa and Rebane, 1985; Kwa et al., 1994b). The values found for the activation energy and promoting mode frequency of intersystem crossing differ from those found for internal conversion. The very small negative activation energy for intersystem crossing may be explained by an intersection of the potentials of the S_1 and the T_0 state in the minimum of the S_1 potential. Since the T_0 state is about $10,000 \text{ cm}^{-1}$ lower in energy than the S_1 state and the S_1 - S_0 energy difference is $14,600 \text{ cm}^{-1}$, this can be achieved by placing the T_0 potential between the S_1 and S_0 potentials, or by displacing the potential T_0 even more horizontally as the S_1 potential energy surface, see Fig. 11, a and b. The latter configuration suggest that upon formation of a triplet state in the CP47 complex, a “new” electronic state is formed which is shifted along the nuclear coordinates. This situation may be encountered when the interactions of a multimeric complex are broken and the triplet resides on one of the monomer compounds of the multimer. This is similar to what was concluded from the spectral changes observed upon triplet formation.

The 350 cm^{-1} mode responsible for the increase of the internal conversion with temperature is considerably lower than that usually found active in aromatic hydrocarbons in vapor ($\sim 3000 \text{ cm}^{-1}$; Engman and Jortner, 1970; Avouris et al., 1977). This may not be very surprising since the coupling to low-frequency vibrational modes to the electronic states of chlorophyll molecules in a micelle of detergent and especially in a protein matrix is likely to be different from

non-heme organic molecules in a vapor; furthermore, the electrostatic interaction between pigments in dimeric or oligomeric structures may get distorted in the excited state, leading to an enhancement in the overlap of the Franck-Condon factors for low frequency intramolecular and intermolecular modes or both. For instance, for the BChl *a* dimer of the special pair of the bacterial RC it is now well established that a set of low frequency modes in the range $30\text{--}200 \text{ cm}^{-1}$ couple strongly to the first excited singlet state (Shreve et al., 1991; Vos et al., 1993). Also internal conversion from this excited state to the ground state is most likely strongly temperature-dependent (Nagarajan et al., 1993).

Lowest energy level in CP47

From the 4 K T-S spectra (Fig. 5, c and d) we conclude that the lowest state(s) in the CP47 complex lies at $\sim 685 \text{ nm}$ and gives rise to fluorescence at 690 nm . Since this state is lower in energy than the primary donor P680 in the PS II RC, the CP47 lowest states may be expected to trap a significant fraction of the excitations in larger complexes as CP47-RC or PS II core particles at low temperature. The thermal energy, $k_B T$, is comparable to the energy barrier between the CP47 lowest state and P680 at $\sim 125 \text{ K}$ (assuming the main fraction of P680 absorbs at 681 nm). Thus, below this temperature the interpretation of spectroscopic data ascribed to the triplet state of P680 will be complicated by the formation of triplet states of these trap-pigments. Only a careful deconvolution of the T-S spectra as a function of temperature will allow the determination of the true T-S spectrum of P680 in PS2 cores and membrane fractions.

We are grateful to Camiel Eijkelhoff and Florentine Calkoen for the expert preparation of the CP47 particles and Prof. R. S. Knox for valuable comments regarding the application of the theory of non-radiative decay. This work was supported by the Netherlands Organization for Scientific Research (NWO) via the Dutch Foundations for Physical Research (FOM) and Chemical Research (SON). J.P.D. is supported by a fellowship from the Royal Netherlands Academy of Arts and Sciences (KNAW).

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