Temperature-Dependent Triplet and Fluorescence Quantum Yields of the Photosystem II Reaction Center Described in a Thermodynamic Model

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ABSTRACT   A key step in the photosynthetic reactions in photosystem II of green plants is the transfer of an electron from the singlet-excited chlorophyll molecule called P680 to a nearby pheophytin molecule. The free energy difference of this primary charge separation reaction is determined in isolated photosystem II reaction center complexes as a function of temperature by measuring the absolute quantum yield of P680 triplet formation and the time-integrated fluorescence emission yield. The total triplet yield is found to be 0.83 ± 0.05 at 4 K, and it decreases upon raising the temperature to 0.30 at 200 K. It is suggested that the observed triplet states predominantly arise from P680 but to a minor extent also from antenna chlorophyll present in the photosystem II reaction center. No carotenoid triplet states could be detected, demonstrating that the contamination of the preparation with CP47 complexes is less than 1/100 reaction centers. The fluorescence yield is 0.07 ± 0.02 at 10 K, and it decreases upon raising the temperature to reach a value of 0.05–0.06 at 60–70 K, increases upon raising the temperature to 0.07 at ~165 K and decreases again upon further raising the temperature. The complex dependence of fluorescence quantum yield on temperature is explained by assuming the presence of one or more pigments in the photosystem II reaction center that are energetically degenerate with the primary electron donor P680 and below 60–70 K trap part of the excitation energy, and by temperature-dependent excited state decay above 165 K. A four-compartment model is presented that describes the observed triplet and fluorescence quantum yields at all temperatures and includes pigments that are degenerate with P680, temperature-dependent excited state decay and activated upward energy transfer rates. The eigenvalues of the model are in accordance with the lifetimes observed in fluorescence and absorption difference measurements by several workers. The model suggests that the free energy difference between singlet-excited P680 and the radical pair state $P680^+I^-$ is temperature independent, and that a distribution of free energy differences represented by at least three values of about 20, 40, and 80 meV, is needed to get an appropriate fit of the data.

INTRODUCTION

Charge separation in the photochemical reaction center (RC) of PS II involves ultrafast electron transfer from a Chl a molecule called P680 to a Pheo a molecule designated I (Seibert, 1993). The free energy difference between the singlet-excited state of the primary electron donor (P680*) and the radical pair state $P680^+I^-$ is an important parameter of charge separation and stabilization. It was first pointed out by Van Grondelle (1985) and Van Gorkom (1985) that there is a dynamic equilibrium between excited state and radical pair. Based on these ideas, Schatz et al. (1988) presented a kinetic model, the so-called exciton-radical pair equilibrium model (see also Fig. 1), which explained the strong biphasic excited state decay in PS II core particles quite well (Schatz

et al., 1987) and yielded a value for the free energy difference of charge separation in those particles.

The exciton-radical pair equilibrium model has been used by a number of authors to calculate the free energy difference of charge separation in isolated PS II RC preparations (Booth et al., 1990, 1991; Roelofs et al., 1991). This was done by determining the intrinsic charge separation rate and the lifetime and yield of the longest-lived fluorescence decay components, ascribed to recombination luminescence. It was found that the free energy difference ΔG of charge separation is strongly temperature-dependent (Booth et al., 1990, 1991; Roelofs et al., 1991), similar to the results of Woodbury and Parson (1984) on reaction centers of *Rhodobacter sphaeroides*. Below 180–200 K, an almost linear dependence of ΔG on temperature was found, which was ascribed to a large entropy contribution to ΔG.

In these types of calculations two main difficulties arise. First, only the longest fluorescence lifetimes in the tens of nanoseconds time-range were ascribed to recombination luminescence in the studies cited above. The excited state decay of PS II RCs is strongly multiexponential with decay components in the 100-fs to 100-ns time range, with nearly identical emission spectra. Therefore it cannot be excluded that recombination luminescence also occurs with shorter lifetimes, especially because fluorescence kinetics of well-stabilized preparations revealed a 6-ns component with an

Abbreviations used: Chl, chlorophyll; FWHM, full width at half maximum; I, primary electron acceptor of photosystem II; P680, primary electron donor of photosystem II; Pheo, pheophytin; PS, photosystem; RC, reaction center, T − S, triplet-minus-singlet.
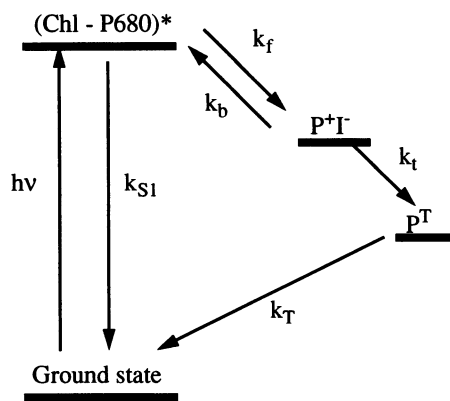
FIGURE 1 Schematic representation of the exciton-radical pair equilibrium model for the primary charge separation process in PS II. The model assumes ultrafast equilibration of excitation energy. The rate constants k_f and k_b describe the equilibrium between the primary radical pair P^+I^- and singlet excited Chl state, k_t represents singlet-triplet mixing and decay of the triplet radical pair to the triplet state of P680, k_T describes the decay of P680^T to the groundstate, and k_{SI} represents the sum of decay processes of singlet excited Chl to the groundstate.

identical spectrum to that of recombination luminescence (Roelofs et al., 1993). In addition, the yields of a such long-lived components are hard to determine because the amplitudes are much smaller than those of the components in the 10-ps to 1-ns range. Second, the antenna pigments in the RC are not taken into account, although they will play a temperature-dependent role in trapping and excited state decay.

In a previous paper, we reported preliminary triplet quantum yield measurements as a function of temperature in a complex of the PS II RC and the core antenna protein CP47 (Van Kan et al., 1992). The results were analyzed in terms of the exciton-radical pair equilibrium model and yielded a temperature-independent free energy difference of about −50 meV for the charge separation reaction. In this contribution we report the quantum yields of radical pair formation monitored by the Chl triplet yield and fluorescence as a function of temperature in isolated PS II RC particles, to analyze the equilibrium between the excited and radical pair states as a function of temperature. Based on the results we propose an extended version of the exciton-radical pair equilibrium model by including a specific energy distribution of the RC electronic states and including the temperature dependence of activation rates and internal conversion. The model is only consistent with the experimental data if a distribution of charge separation driving forces is assumed. The model globally predicts the observed decay rates and yields temperature-independent values for the free energy difference of charge separation.

MATERIALS AND METHODS

PS II RC complexes were isolated from spinach by means of a short Triton X-100 treatment as described earlier (Kwa et al., 1992a). Spectroscopic characteristics of these preparations have been reported by Kwa et al. (1992a, 1994b, c) and Roelofs et al. (1993). The method is essentially similar

to the procedure reported by Van Leeuwen et al. (1991), which results in preparations binding six Chl a and two Pheo a molecules and identical 4-K absorption spectra (Van Leeuwen, 1993, Kwa et al., 1994b). Samples used for flash-induced absorption difference experiments were diluted in a buffer containing 20 mM BisTris (pH 6.5), 20 mM NaCl, 0.03% n-dodecyl-β,D-maltoside and 80% (w/v) glycerol to an optical density of 0.5–0.7 at 675 nm. Samples used for fluorescence experiments were diluted in the same buffer to an optical density of 0.05–0.10 at 675 nm. An Oxford Instruments F1204 (Oxford, UK) helium flow cryostat in conjunction with an Oxford ITC-4 temperature controller were used to vary the temperature.

Laser-flash induced T − S absorption difference spectra were measured on a home-built single-beam spectrophotometer described in more detail by Van Kan et al. (1992). The excitation laser flashes (FWHM ∼8 ns) were at 610 nm with 1-Hz frequency. Decay-associated spectra were estimated from a global analysis (Knutson et al., 1983, Van Stokkum et al., 1994) of kinetic traces recorded at 40–80 probe wavelengths. Absolute values for triplet quantum yields were obtained from the slope of the saturation curves at zero excitation intensity using free Chl a in Triton X-100 at 77 K (Kwa, et al., 1994a) as a calibration standard. For the calculation of the PS II RC triplet quantum yield a quantum yield of 0.64 of free Chl a was assumed (Bowers et al., 1967). In addition, it was assumed that the bleaching in the T − S spectrum corresponds to the disappearance of the oscillator strength of one Chl (i.e., P680 is either a monomer or a multimer in which the oscillator strength of one Chl disappears upon triplet formation, which is justified by the evidence that the triplet resides on a single Chl molecule; see Seibert, 1993).

The emission spectra were recorded on a home-built steady-state fluorimeter described and corrected for the sensitivity of the system as in Kwa et al. (1992a). 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 absolute fluorescence yield was obtained by integrating the emission spectrum from 650 nm to 750 nm and comparing the yield with that obtained with free Chl-a in Triton X-100 at 77 K, for which a quantum yield of 0.30 was assumed (Seely et al., 1986).

RESULTS

Characterization of triplet states

In the RC of PS II, the 610-nm laser excitation used for the experiments described below generates the radical pair state P680$^+$I$^-$. Because of the absence of secondary electron transfer this state has a long lifetime, which causes hyperfine induced singlet-triplet mixing to take place to a relatively large extent and the triplet radical pair state (P680$^+$I$^-$)^T to be generated (see also Fig. 1). Charge recombination of this triplet radical pair state will result in the triplet excited state of P680 (P680^T). Since P680^T is formed via the radical pair mechanism, its quantum yield directly reflects the quantum yield of P680$^+$I$^-$.

Fig. 2 a shows a characteristic trace of the laser flash-induced absorbance difference at 681 nm at 4 K. Global analysis of such traces recorded between 650 and 710 nm revealed that the decay to the ground state is biphasic with lifetimes of 1.6 and 6.6 ms in a 2:1 amplitude ratio (Fig. 2 c) with almost identical spectra (Fig. 2 b) in which the maximal bleaching is at 681 ± 1 nm. The FWHM of the spectra is 6.5 nm, and the slope of the spectra on the red side is less steep than on the blue side, in accordance with earlier 4-K T − S difference spectra (Van Kan et al., 1990; Kwa et al., 1994b).
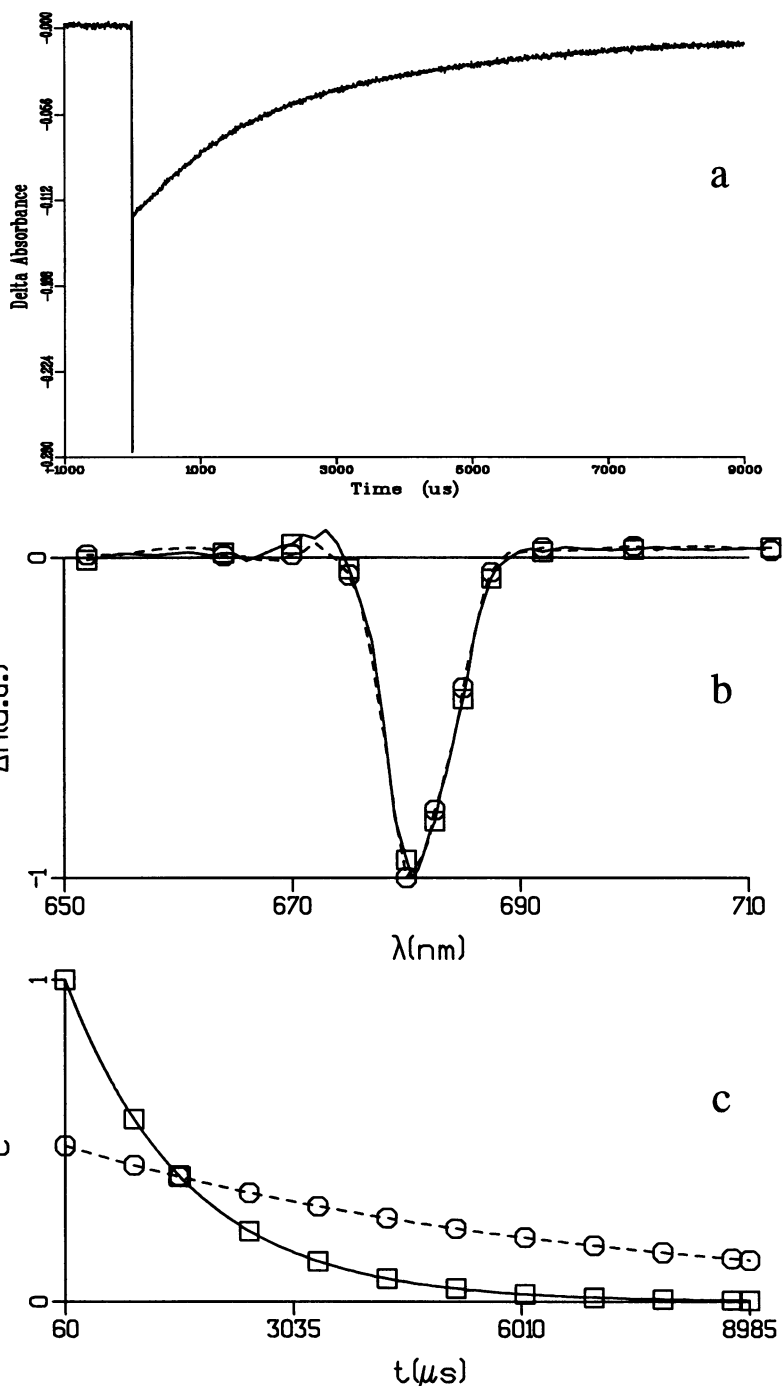
FIGURE 2 (a) Time course of the triplet in PS II RC preparations, induced by repetitive (1 Hz) laser excitation at 4 K, recorded at 680 nm. The recordings are the average of 32 measurements. (b) Decay-associated spectra-obtained from a global analysis of traces at 4 K as in (a), recorded at 50 wavelengths between 650 and 710 nm. (c) Kinetic vectors of the decay-associated spectra, described by decay rates of 1.6 ms (squares) and 6.6 ms (circles). Both components have their maximum bleaching at 681 ± 1 nm, with FWHM 6.5 nm. Excitation density: 4 photons/RC.

Above 40 K only one kinetic component could be resolved in the triplet decay. The decay rate increases linearly from $(1.9 \text{ ms})^{-1}$ to $(1.3 \text{ ms})^{-1}$ upon raising the temperature from 40 K to 200 K (Fig. 3). This is in reasonable agreement with the lifetime of 1.0 ms observed at 270 K (Durrant et al., 1990; Yruela et al., 1994). For an explanation of the biphasic decay at low temperature we suggest that at 4 K the decay from the individual triplet sublevels is resolved. The observed decay times and temperature dependence of the triplet decay kinetics may well be explained by this mechanism (see also Searle et al., 1990). However, as will be shown below, not

only the triplet state of P680 will be formed via the radical pair mechanism, but also to some extent Chl and Pheo antenna triplets via intersystem crossing of Chl* or Pheo*. Apparently the spectra of the latter triplets do not differ much from the P680 triplet, as it is not possible to distinguish the two types of triplets by global analysis. Therefore, we will restrict ourselves to the total triplet yield in the remaining part of this paper.

The (77 K) T − S spectrum in the Soret region was measured to check for Pheo and carotenoid triplet states (Fig. 4). Besides typical bands of Chl triplets (with an absorption
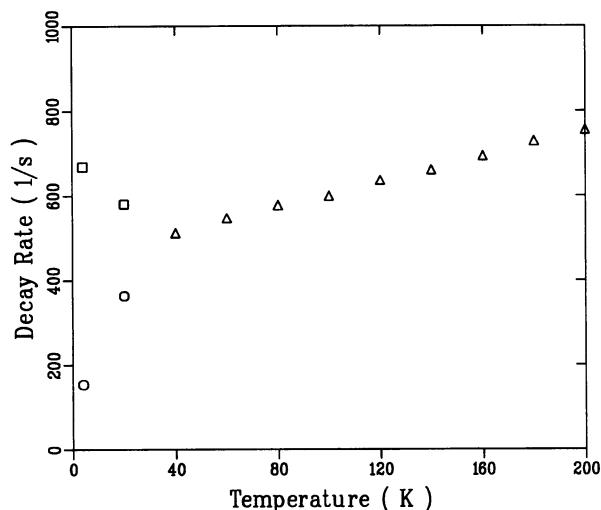
FIGURE 3 Triplet decay rates as a function of temperature, obtained by global analysis of kinetic traces as in Fig. 2. Above 40 K only one component was resolved.

increase at 465–470 nm and decreases at ~410 nm and ~435 nm), a small but significant bleaching at 545 nm is observed (see the inset of Fig. 4 for spectra at 4 K and 40 K). We attribute this bleaching to the disappearance of a Pheo-Q_x transition. From the amplitude of the bleaching and the absorption spectrum of Pheo a in Triton X-100 (Kwa et al., 1994c) it can be deduced that the contribution of the Pheo a triplet state to the bleaching in the Q_y region is 0.06. This is in accordance with earlier results of Van der Vos et al. (1992), who determined the Pheo^T yield to be 0.05–0.10 and of Kwa et al. (1994c) and Tang et al. (1990), who show that the Q_y transition of the Pheo a molecule that forms a triplet state is practically degenerate with P680. We checked for β-carotene triplet states, but could not detect any. In CP47 (and CP47RC) they are easily detected (Groot et al., unpublished observations), which suggests that, at least at temperatures below 77 K, no triplet transfer from Chl to carotene takes place and that there is no measurable contamination of the RC samples with CP47 (<1/100 RC particles). It is important to mention that the absence of carotenoid triplet states implies that the determined Chl triplet quantum yields are total triplet quantum yields.

Absolute quantum yield of triplet states

Fig. 5 shows the triplet quantum yield as a function of temperature. The yield is 0.83 ± 0.05 at 5 K, stays more or less constant in the 5–50 K range, and above 50 K decreases gradually to 0.30 at 200 K. This is in reasonable agreement with triplet yields of about 0.30 at 4°C determined by Durrant et al. (1990) and of 0.80 at 10 K and 0.23 at 276 K by Takahashi et al. (1987).

Qualitatively, the temperature dependence of the triplet yield can be understood within the context of the radical pair-exciton equilibrium model in its simplest form (Fig. 1). The equilibrium between P680* and P680$^+$I$^-$ is determined

by the free energy difference ΔG and the temperature. At low temperatures recombination of the radical pair state to the excited state is hard because the energetic barrier is high. Consequently, the P680 triplet yield is high. At higher temperatures the thermal energy becomes sufficiently large to cross the barrier between P680$^+$I$^-$ and the excited state, so an additional decay channel via the excited state is opened and the yield of P680^T is lowered. The T-dependence of the decay rate above 40 K is linear, which assures that there is no additional, activated decay from P680^T other than directly to the groundstate.

Quantum yield of steady-state fluorescence

In the simple form of the radical pair-exciton equilibrium model there is a reverse relation between triplet yield and fluorescence yield. When the equilibrium shifts to the (Chl-P680)* state one expects an increase in the fluorescence yield and a concomitant decrease of the triplet yield. We determined the quantum yield of the total emission as a function of temperature to see whether the reverse relation holds.

Fig. 6 shows emission spectra measured between 10 and 270 K upon 610-nm excitation. The 10 K spectrum peaks at 684 ± 2 nm, with a FWHM of 9.5 nm. The shape of the spectrum hardly changes between 10 K and 75 K, but between 75 K and 270 K the emission spectrum shifts gradually to 682 nm and broadens to an FWHM of 16.5 nm. The broadening is explained by the temperature-dependent equilibration of excitations over all RC pigments. The spectra contain a small contribution of uncoupled Chl at 672 nm. Its amplitude is 3.5% compared with the contribution at 684 nm. Given that the fluorescence yield of monomeric Chl a is 0.30 (Seely, et al., 1986) and that of the main emission of the RC at 684 nm is 0.07 (see below), it probably arises from about 1% of the pigments. This very small contribution is neglected in this paper.

Fig. 7 shows the quantum yield of the total fluorescence (the integral of the spectrum from 650–750 nm) as a function of temperature. The absolute fluorescence yield at 10 K was determined to be 0.07 ± 0.02. It appears that only between ~80 K and ~160 K the reverse relation with the triplet quantum yield is observed. In this temperature range the fluorescence yield increases upon raising the temperature, whereas the triplet yield decreases. Above 160 K the fluorescence yield decreases again, whereas below 80 K a pronounced increase is observed. A maximum in the fluorescence at ~160 K confirms earlier results of Booth et al. (1990); although these authors found the decrease at higher temperatures to be somewhat less pronounced. A maximum in the fluorescence yield at 200 K has also been observed for RCs of *Rb. sphaeroides* by Woodbury and Parson (1984). A similar decrease in the fluorescence yield upon raising the temperature above about 160 K has also been observed in the isolated core antenna complex CP47 (Groot et al., unpublished observations), but only to a minor extent with isolated Chl (Groot et al., unpublished observations). Therefore, we conclude that the decrease of the yield above 150 K is a

FIGURE 4 T − S absorbance difference spectrum from 370–570 nm at 77 K, constructed from the averaged amplitude 50–500 μs after the flashes. *Inset*: enlargement of Pheo Q_x region at 4 K (*solid line*) and 40 K (*dashed line*).
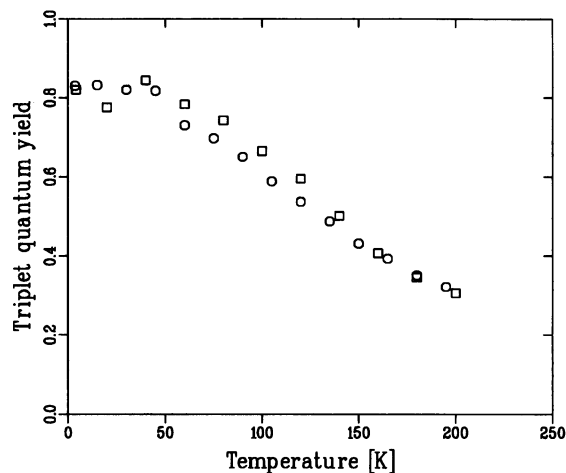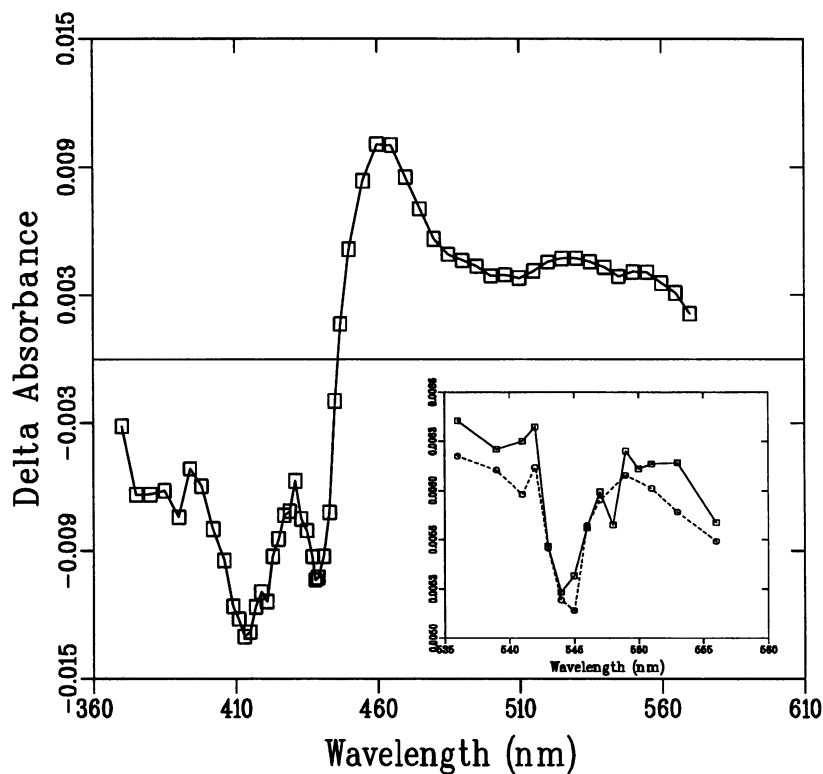
FIGURE 5 Absolute triplet quantum yields as a function of temperature, measured from 4 to 200 K (*circles*) and from 200 to 4 K (*squares*). The yields were calculated from the slope at zero intensity of saturation curves taken at 681 nm (see Material and Methods for details).
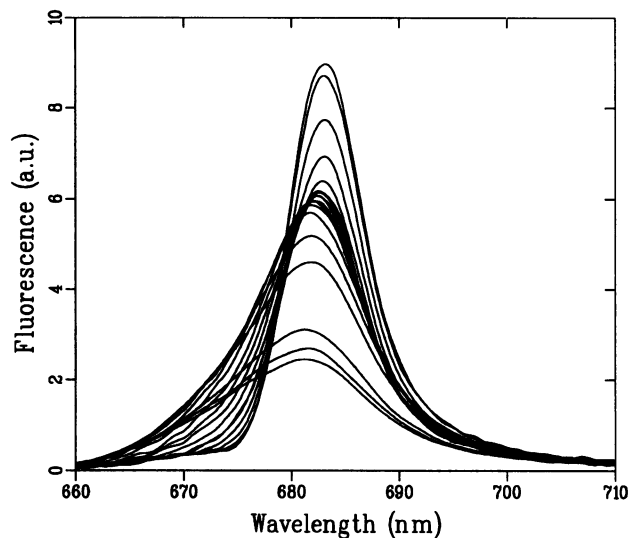
FIGURE 6 Fluorescence emission spectra of PS II RC particles recorded at temperatures from 4–270 K in intervals of 15 K. The emission maximum at 4 K is at 684 ± 2 nm, FWHM 9.5 nm; above 70 K the spectra shift and broaden to 682 nm and 16.5 nm at 270 K. The emission around 670 nm is due to a minor contamination of Chl-*a* pigments uncoupled from charge separation, which we estimate to be less than 1% of the pigments (see text).

general characteristic of Chl-protein complexes. It is very likely caused by quenching of the excited state, which is caused by an increase of the internal conversion rate with increased temperature.

The increase in fluorescence yield upon lowering the temperature from 80 K to 10 K is not observed in CP47 or in free Chl in detergent (Groot et al., unpublished observations). It is also not expected within the context of the simple radical pair equilibrium model (Fig. 1). The increase must be explained by one or more pigments in the RC that trap exci-

tation energy and, at low temperatures, are unable to transfer their energy to P680 causing the excited state to decay via fluorescence, intersystem crossing to the triplet state, or internal conversion. Thus, the present results show that there are antenna pigments at similar or lower energy than P680 in the PS II RC.
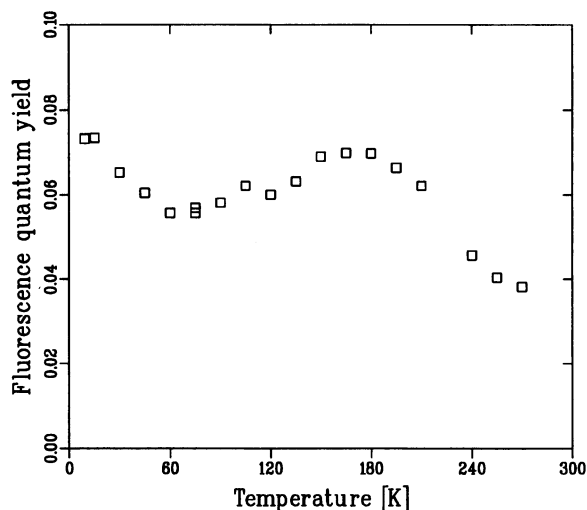
FIGURE 7 Fluorescence quantum yields as a function of temperature obtained by integrating the spectra of Fig. 6 from 650–750 nm and calibrated with free Chl a.

## DESCRIPTION OF A THERMODYNAMIC MODEL

We will simulate the temperature dependence of the triplet and fluorescence yields (Figs. 5 and 7) by a model that describes the kinetics and energetics of charge separation in the PS II RC complex. The model is shown in Fig. 8. It starts from a set of simple physical assumptions and incorporates known values for the parameters required for the description of charge separation. Temperature dependence is introduced in the model via activated upward energy transfer rates and

the internal conversion rate. From this *ab initio* approach we will try to find a value for the free energy difference between P680* and P680$^+$I$^-$. The steady-state solution will be tested by solving the model kinetically and comparing the eigenvalues with rates determined in time-resolved fluorescence and absorption difference experiments by several groups. In the following section we will discuss various aspects of the model.

Excited state decay parameters

The decay of the excited state of Chl is determined by the sum of rate constants for radiative decay (k_R), intersystem crossing to the metastable triplet state (k_{ISC}), and internal conversion into heat (k_{IC}). For free Chl a, k_R can be calculated with the Strickler-Berg relation or from the quantum yield of fluorescence (0.30) and the excited state lifetime (6 ns) (see Seely et al., 1986), i.e., $k_R \sim 0.05$ ns$^-$. The ratio of k_{ISC} to k_R is about 2:1 (Seely et al., 1986), i.e., $k_{ISC} \sim 0.1$ ns^{-1}. When Chl a is connected to a protein, the internal conversion is often stronger. For example, in CP47 k_{IC} has been estimated to be $5 \times k_R = 0.25$ ns^{-1} at 4 K (Groot et al., unpublished observations). This increase probably arises from interactions with the protein that facilitate the conversion of excitation energy into vibrational (phonon) energy or heat. Also in aggregates of pigments the rate of internal conversion is generally enhanced (Alden et al., 1992). The internal conversion is temperature dependent; at high temperature the density of phonon states is larger and the rate increases. This has been described with an energy gap law-type expression by Englman and Jortner (1970). In the strong coupling limit

FIGURE 8 Four-compartment exciton-radical pair equilibrium model for the primary exciton equilibration and charge separation reactions in isolated PS II RC preparations. The levels denoted C671*, C681* and P680* represent the singlet excited states of antenna pigments absorbing around 671 nm and 681 nm and of P680, respectively. The singlet excited states are in contact with each other via the energy transfer rates k_{1-6} and decay with the sum of the loss processes of radiative decay, internal conversion and intersystem cossing $(k_R, k_{IC}$ and k_{IS}, respectively). The level P680$^+$I$^-$ represents the radical pair state which can recombine to the excited state, the ground state or via S–T mixing to P680^T.
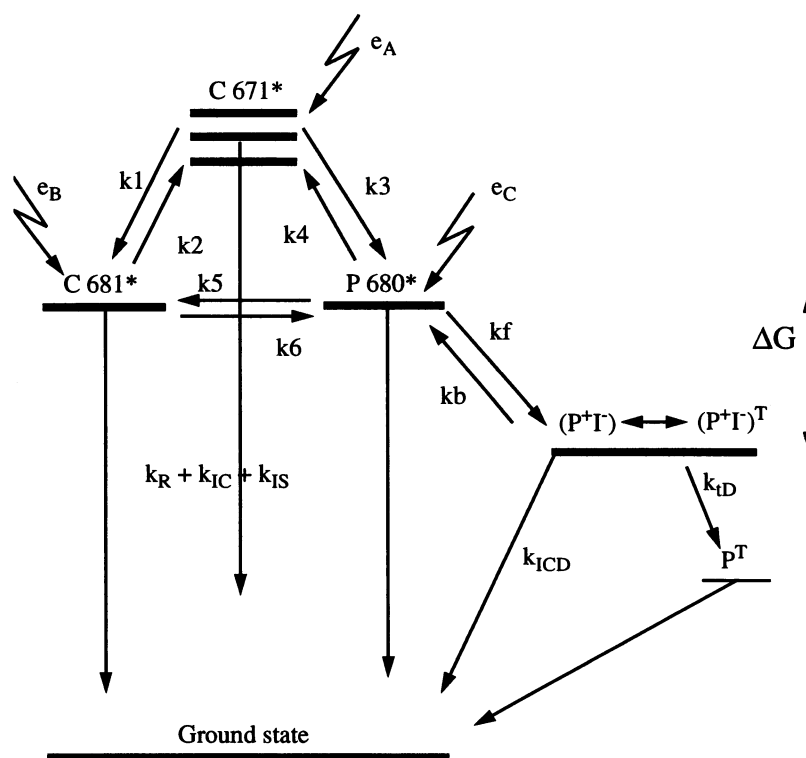
the rate is found to depend on the frequency of the promoting mode, the activation energy, and a factor concerning the rates for radiative decay and intersystem crossing.

To avoid the additional freedom that a fit to this expression would introduce, we have used the frequency of the promoting mode (350 cm^{-1}) and activation energy (650 cm^{-1}) that we obtained from a fit of the temperature dependence of the excited state decay as was measured by us in the CP47 antenna complex (Groot et al., unpublished observations) to the energy gap law expression. It will be shown below that this approximation yields a reasonable but not perfect fit in the higher temperature range.

Energy levels

In the simplest form of the model the distribution of the energy levels of pigments in the PS II RC is simplified by dividing the RC into two parts containing groups of pigments absorbing ~671 and ~681 nm, respectively. The latter group also includes P680. This distribution is consistent with the absorption spectrum in the Q_y region. The average energy difference between these two pigment groups is ~28 meV (see the scheme in Fig. 8). To account for the fluorescence observed at 4 K, an additional trap for excitation energy different from P680 is included in the model. The excited state of the pigment(s) constituting this trap will in part decay via intersystem crossing to the metastable triplet state, which will be included in the T − S spectrum (Fig. 2 b). In Fig. 8 the levels containing the 671- and 681-nm absorbing accessory pigments are denoted C671 and C681, respectively. Inasmuch as the excitation was at 610 nm in the vibrational sub-bands, all pigments have about equal probability of being excited, and the levels are populated according to their relative oscillator strength with the excitation densities ϵ_{C671}, ϵ_{C681}, and ϵ_{P680}.

From the temperature dependence of the fluorescence yield it is evident that at T ~ 40 K C681 becomes thermally connected to the other pigments. Because P680 is included in this set of other pigments, the fluorescence yield decreases. At this temperature the rate to escape from C681 must roughly be of the same order as the excited state lifetime of C681, which is expected to be 1–5 ns. This points to slow energy transfer between P680 and C681. Though energy transfer between P680 and C681 can be relatively slow, e.g., because the pigments are separated by a large distance, energy transfer will still be one or two orders of magnitude faster than excited state decay (at least in the hundreds of picoseconds time range). This means that the activation energy to escape from C681 corresponds to the thermal energy $k_B T$ at T ~ 100 K or to a spectral energy difference of ~3 nm to make the effective rate of energy transfer (the product of relative oscillator strength, energy transfer rate, and Boltzmann term, see Energy Transfer Rates, below) comparable to the excited state decay rate around 40 K. Consequently, escape from the state C681* occurs via an energy level or pigment absorbing around 678 nm.

Generally, the energies of pigments in a protein matrix are inhomogeneously distributed, which, for instance, implies that the $S_0 \rightarrow S_1$ transition of P680 is almost always at either somewhat lower or higher energy than of C681. It has also been shown that P680 is heterogeneous: a shoulder in the spectrum of P680 at 683 nm has been ascribed to the presence of a second spectral form (Otte et al., 1992), and Van der Vos et al. (1992) even observed three spectral forms by absorption detected magnetic resonance spectroscopy. Recently, Kwa et al. (1994b) found by high-resolution absorption difference spectroscopy two species absorbing at 680 and 684 nm in a ratio of 1:0.6. We have included the inhomogeneity/heterogeneity of P680 and C681 in the model by taking into account that there are certain fractions of RCs in which C681 is either lower or higher in energy than P680. For simplicity, we calculated the model only in two energetic states, i.e., the state in which P680 is 3 nm higher in energy than C681, and the state in which the energy of P680 is the same as that of C681. A lower energy of P680 gives the same result as the latter situation because of fast charge separation once the excitation arrives at P680, so the latter state includes the possibility that P680 is, for example, as low as 683 nm. Note that the former configuration generates the above-mentioned decrease in fluorescence between 4 and 50 K.

Assuming that $k_{ISC} = 2 k_R$ as for monomeric Chl a, the C681 triplet yield can be calculated to be 0.15 at 4 K. With 5–10% of the decay of P680$^+$I$^-$ directly to the singlet ground state (see Volk et al., 1993) this means that ~1.1 × (0.83 − 0.15) = 75% of all excitations finally reach (at 4 K) the radical pair state and about 25% C681. A distribution of the excitations in a ratio of 1:3 over C681 and P680 can be obtained in the model in two ways: the relative energy transfer rates from C671 to C681 and P680 can be varied, and/or the fraction of RCs with C681 as the lowest state can be adjusted.

From the Pheo triplet formation and the results of Tang et al. (1990), Van der Vos et al., (1992) and Kwa et al., (1994c) where it is shown that at least one of the $Q_{y(0-0)}$ transitions of the two Pheos is degenerate with P680, it is clear that one of the Pheo pigments may be involved in the low-temperature trap C681. The triplet yield of the Pheo pigment is about 0.06 in our experiments, and from the ratio of the rates of radiative decay and intersystem crossing (see above) we can calculate that the fluorescence emission of the Pheo pigment must have a yield of 0.03. This means that Pheo only is not sufficient to constitute the trap in order to account for the Φ_F of 0.073 at 4 K. Also considering the results of Kwa et al. (1994c), who showed that the emission at 4 K mainly arises from a Chl pigment, it is most likely that the trap consists of one Chl a pigment and one Pheo a pigment. Thus in the "final" models, two Chls contribute to P680, one Chl and one Pheo to C681, and the remaining Pheo and three Chls to C671. Because the extinction coefficient of the Q_y transition of Pheo a is about 0.6 of Chl a, we assumed therefore relative oscillator strengths of 1.6/7.2 for C681, 2/7.2 for P680, and 3.6/7.2 for C671.

Energy transfer rates

In the model (Fig. 8) all levels are connected with each other through a set of energy transfer rates k_1 to k_6. The transfer rates are expressed in the parameter k_{et} (see Table I) multiplied with the probability of a transition to a particular level, which is expressed in terms of relative oscillator strengths. This makes k_{et} identical to the average lifetime of one pigment. All upward rates are assumed to be activated with a Boltzmann term, $\exp[-\Delta E/k_B T]$, where ΔE is the energy difference between two levels. Thus the energy transfer rate from a level A to a level B, where A is lower in energy than B, is given by $k_{A \to B} = \epsilon_A k_{et\,A,B} \exp[-(E_B - E_A)/k_B T]$. The rate $k_{et\,5,6}$ (together with $\Delta E_{678-681}$) determines the temperature dependence of the kinetics of C681. It must be chosen slow enough to enable the excited state decay rate to compete with it at low temperature, but it still is an energy transfer rate, so it cannot be too slow. In the simulations it will be shown that a best result is obtained using a value of $\sim(300\ \text{ps})^{-1}$.

Charge separation and recombination

Charge separation (k_f) occurs from level P680* to level P680$^+$I$^-$ in a few (tens of) picoseconds. The backward rate (k_b), representing charge recombination to the excited state, is set equal to the forward rate multiplied with the Boltzmann factor $\exp[-\Delta G/k_B T]$, in which ΔG is constant and independent of temperature. It is found (see below) that realistic simulations only are found when a distribution of ΔG values is used. The distribution is approximated by a discrete set of ΔG values, in the present calculations at most three. In this case the calculations are performed separately for each ΔG and averaged, so each ΔG has equal weight.

The radical pair state may also recombine directly to the ground state with the rate k_{ICD}. This rate is probably very slow

TABLE 1 Parameters used for simulation, where A = C671, B = C681, C = P680, and D = P680$^+$I$^-$. The actual energy transfer rate from a level X to a level Y, where X is lower in energy than Y, is given by:
$k_{X \to Y} = \epsilon_X k_{et\,X,Y} \exp[-(E_Y - E_X)/k_B T]$.

Parameters	Model I Rates (ns^{-1})/ Energy (meV)	Model II Rates (ns^{-1})/ Energy (meV)
$\epsilon_{A,B,C}$	3.6/7.2, 1.6/7.2, 2.0/7.2	3.6/7.2, 1.6/7.2, 2.0/7.2
$k_{et\,1,2}$	20.000	100
$k_{et\,3,4}$	135	100
$k_{et\,5,6}$	3	2
k_f	200	300
$k_{R\,A,C}$	0.05	0.05
$k_{ISC\,A,C}$	2.2 k_R	2.2 k_R
$k_{IC}(T = 0)_{A,\,C}$	7.5 k_R	7.5 k_R
$k_{R\,B}$	0.05	0.05
$k_{ISC\,B}$	2.4 k_R	2.2 k_R
$k_{IC}\,(T = 0)_B$	1.1 k_R	1.0 k_R
k_{tD}	0.01	0.01
$k_{IC}\,(T = 0)_D$	0.0005	0.0005
$E_{C671-681}$	28	28
$E_{C671-678}$	20	20
$E_{C678-681}$	8	8
$\Delta G_{1,2,3}$	20, 40, 80	25, 40, 80

(Volk et al., 1993). Therefore, we included $k_{ICD} = 0.5\ \mu\text{s}^{-1}$ and the temperature dependence for internal conversion processes as discussed above. The kinetics of P680$^+$I$^- \to$ P680^T are described in the model by a rate constant $k_{t,D}$. Formally this is not correct: the singlet-triplet mixing between the states P680$^+$I$^-$ and (P680$^+$I$^-$)^T should be described by the appropriate quantum dynamics (see Volk et al., 1993), and only the state (P680$^+$I$^-$)^T recombines to P680^T. The overall rate is determined by the bottleneck process of singlet-triplet mixing, which is much slower than the triplet radical pair recombination rate (Volk et al., 1993). For $k_{t,D}$ we take the longest lifetime of the radical pair observed in absorption difference measurements (Van Kan et al., 1990; Volk et al., 1993), i.e., $k_{t,D} = 0.01\ \text{ns}^{-1}$.

Currently, there is a debate in the literature concerning the kinetics of primary charge separation in the PS II RC. The group of Klug and co-workers favors ultrafast equilibration of excitation energy and a "slow" charge separation in about 20 ps (Durrant et al., 1992, 1993; Hastings et al., 1992), whereas other groups favor "fast" charge separation in about 2–3 ps and slow energy transfer (Wasielewski et al., 1989a, b; Jankowiak et al., 1989; Roelofs et al., 1991; Schelvis et al., 1994). In the following section, we will present simulations for both types of situations in models I and II, respectively.

SIMULATIONS

The parameters discussed above may all have a certain temperature dependence but this is most likely much weaker than the temperature dependence of the activated rates and the excited state decays that we have included and are therefore less important for the model. Using the parameters described above, the model is solved (see Appendix) for the total emitted fluorescence yields of C671, C681, and P680 between 4 and 270 K, and the triplet yields of C681 through intersystem crossing and P680 through the radical pair mechanism. The sum of these corresponds to the triplet yield extracted from the absorbance difference measurements at 681 nm.

In Fig. 9 simulations of the fluorescence (a) and triplet (b) yield data are shown (model I). The rate of excitation equilibration between C671 and C681 in the simulation was assumed to be subpicosecond ($10^4\ \text{ns}^{-1}$) following results from transient absorption experiments by Durrant et al. (1992). Transfer between C671 and P680 was assumed to be 135 ns^{-1} (the actual value of this parameter is not that important for the present simulation; it produces however, eigenvalues that are in line with observed values; see below) and the intrinsic charge separation rate was taken as 200 ns^{-1}, following Wasielewski et al. (1989a, b), Jankowiak et al. (1989), Roelofs et al. (1991), and Schelvis et al. (1994). We note that a charge separation rate of 50 ns^{-1}, suggested by the findings of Hastings et al. (1992) and Durrant et al. (1993), produces an equally good simulation. The fraction of centers with C681 as the lowest state was 0.33.
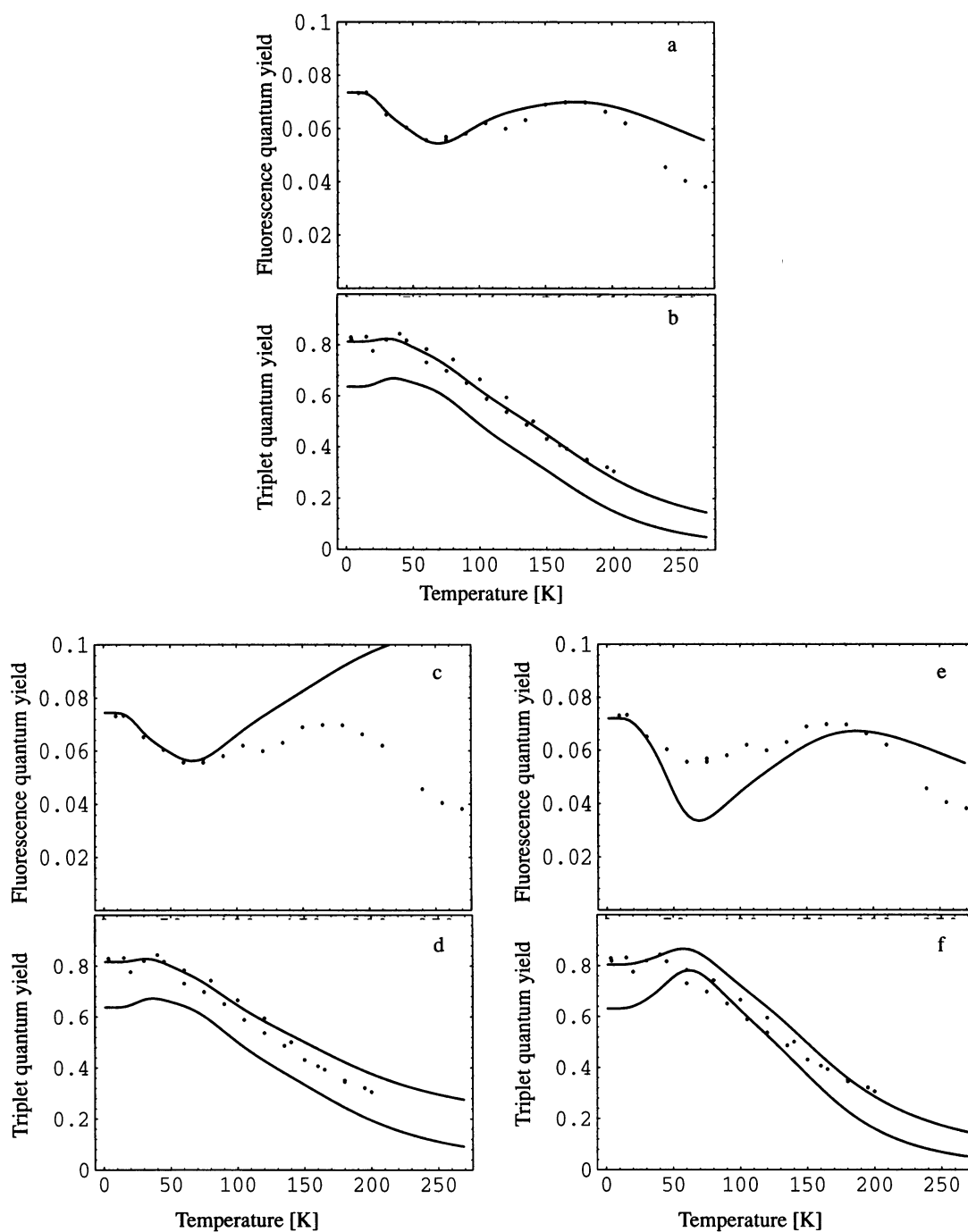
FIGURE 9   Simulation of the temperature dependent fluorescence yield (a), the P680 triplet yield (b, lower curve) and the triplet yield of C681 and P680 (b, upper curve), yielding ΔG_1 = 20 meV, ΔG_2 = 40 meV and ΔG_3 = 80 meV. See for parameters used Table I, model I. (c and d): Simulation using the parameters of Table I, model I but with k_{IC} = constant. (e and f): Simulation using ΔG_1 = 35 meV, ΔG_2 = 70 meV.

The simulation using these parameters (see also Table 1) is satisfactory and yields ΔG_1 = 20 meV, ΔG_2 = 40 meV and ΔG_3 = 80 meV. It can be seen that at low temperature the Φ_F curve is well described by the inclusion of C681 in the model. The simulations deviate at $T > 200$ K; apparently the temperature dependence of the internal conversion in the RC is not identical to that in the CP47 antenna. However, using this temperature dependence is quite an improvement on treating k_{IC} as a constant

(Fig. 9 c, d). In Fig. 9 e, f a simulation is shown using only two ΔG's (ΔG_1 = 35 meV, ΔG_2 = 70 meV). This illustrates that at least three ΔG values are required to obtain a reasonable fit of the data.

The simulations do not depend very much on the actual energy transfer or charge separation rates, except for those rates connecting the C681 and P680 levels. The energy transfer rates between C671 and C681 and between C671 and P680 can, for example, also be made equal (100 ns^{-1}). To

produce a good fit (not shown), the fraction of centers with C681 as the lowest state had to be increased to 0.58. For the charge separation rate 3.3 ps^{-1} was used. All other parameters were similar to the ones used in the previous simulation, including the ΔGs needed to provide a good fit: $\Delta G_1 = 25$ meV, $\Delta G_2 = 40$ meV and $\Delta G_3 = 80$ meV.

KINETICS

The energy transfer rates have a more direct effect on the kinetics of the system as reflected by the eigenvalues of the set of kinetic equations. Below we will discuss the eigenvalues of the models at different temperatures and compare them with experimental values.

Table 2 (upper half) shows the eigenvalues for the simulation of model I (Fig. 9 a, b) at four temperatures. The six calculations, with ΔG_{1-3} and either C681 or P680 as the lowest state, result in multiple values for the third and fourth eigenvalue, but have little effect on the other two eigenvalues.

The first eigenvalue, which changes from 130 fs to 220 fs when the temperature is changed from 270 K to 10 K, is associated with equilibration between C671 and C681. The second, a 5-ps phase, is observed predominantly in the decay of P680 and in the rise of the radical pair. At 270 K it can also be observed in the decay of C671 and C681 with an amplitude 40 times smaller than the amplitude of the 130-fs component. Both eigenvalues are direct reflections of the inserted energy transfer and charge separation rates. When the intrinsic charge separation rate is changed to $(20 \text{ ps})^{-1}$, the second eigenvalue is 20 ps at 10 K but decreases to 12 ps at 270 K, because at this temperature energy transfer from P680 back to the antenna pigments competes with charge separation.

The third eigenvalue is associated with C681. At low-temperature C681 decays either through the "unquenched" excited state decay processes or through slow energy transfer to P680. At high temperature, thermal energy is sufficient to cross the barrier to C671 via fast energy transfer, whereupon the equilibrated C671/C681 excited state decays via transfer from C671 to P680. The eigenvalue decreases from 0.9 ns and 4.4 ns at 10 K (two values are obtained, for P680 and for C681 as the lowest state) to 50 ps at 270 K. At 10 K the 4.4-ns component is only found in the decay of C681, whereas the

0.9-ns component is also found in the rise of the radical pair with an amplitude comparable to that of the 5-ps phase. At $T > 75$ K it is observed as the decay rate of the equilibrated antenna; it increases from $(100 \text{ ps})^{-1}$ to $(50 \text{ ps})^{-1}$ at 270 K, which is in fact the time of energy transfer from C671 to P680. The contribution of this component to the rise of the radical pair increases until it is twice as large as that of the 5-ps phase at 270 K (even when C681 and P680 are selectively excited, its amplitude in the radical pair kinetics is still 1/3 of that of the 5-ps component). The amplitude of the third eigenvalue in the kinetics of P680 is diminished because of the subsequent fast charge separation.

The last eigenvalue is actually a set of three values as a consequence of the three values of ΔG that were used. At 10 K they are only present in the decay of the radical pair state. At 77 K the fastest, 20-ns, rate is present in the decay of states C671, C681, and P680 with an amplitude ~100 times smaller than those of the other eigenvalues, because recombination to P680* can occur from the P$^+$I$^-$ state with the smallest ΔG. The 75- and 97-ns components have negligible contribution to the decay of C671, C681, and P680 at this temperature. At higher temperatures the amplitude of the fourth eigenvalue in the decay of the excited state increases, although e.g., at 270 K, the amplitude of the 12-ns component is 10 times smaller than that of the 2-ns component. In turn, this rate has about 2 times lower amplitude in the excited state decay as the 50-ps eigenvalue at this temperature.

The parameters of model II, with slower (10 ps) energy transfer from C671 to C681, produce somewhat different eigenvalues. The first eigenvalue of 3 ps is a direct reflection of the inserted charge separation rate. The second eigenvalue of 20 ps, which is almost independent of temperature, is the time of transfer from high to low energy, i.e., it represents the equilibration time of the antenna system. At 10 K the amplitude of this component in the radical pair rise is 1.5 times larger than that of the 3-ps component. At 270 K they have equal amplitude. The behavior of the third and fourth eigenvalue is practically not influenced by the change in equilibration rate, except that the 100–200-ps decay time is present in the decay of C671 at somewhat higher temperature, because equilibration between C671 and C681 is slower. The third eigenvalue is somewhat slower than the corresponding rate of model I, because the energy transfer rate from C671 to P680 has been changed from 135 ns^{-1} to 100 ns^{-1} in model II. At 270 K, the contribution of this component to the rise of the radical pair is equal to that of the 3-ps and 20-ps components.

DISCUSSION

The temperature dependence of the total emission yield indicates that the reaction center of PS II contains pigments (designated C681) that at 4 K are unable to transfer excitation energy to the primary electron donor P680. When the temperature is raised, the thermal energy of the excitation energy becomes sufficient to cross the energy barrier to P680, which

TABLE 2　Eigenvalues of the model at T = 10, 77, 150, and 270 K for model 1 (upper panel) and model 2 (lower panel).

Temperature [K]	$\tau 1$ (ps)	$\tau 2$ (ps)	$\tau 3$ (ns)	$\tau 4$ (ns)
Model 1				
10	0.22	5	0.93, 4.4	97
77	0.22	5	0.42, 0.58	20, 75, 97
150	0.18	4–5	0.10–0.12	6, 16, 75
270	0.13	3–4	0.050–0.065	2–12
Model 2				
10	3.3	20	1.3, 4.8	97
77	3.2	20	0.83, 1.2	22, 83, 97
150	3.0	19	0.25	7, 20, 75
270	2.5, 3.0	18	0.10	2–12

causes the yield of trapping in the radical pair state to increase and Φ_F to decrease. At about 50 K the thermal energy is sufficient to compete with the smallest ΔG, and the equilibrium is shifted somewhat to the side of the antenna system, resulting in an increase of Φ_F and a decrease of Φ_T. When temperature is increased further, the equilibrium shifts further. At about 180 K the fluorescence yield starts to decrease due to the enhancement of the internal conversion with temperature.

Kinetics

The thermodynamic models presented in Fig. 8 explain the experimentally observed temperature dependences rather well. The discussed models I and II are examples of fast and slow energy transfer, respectively, and both can be made consistent with the experimental results. It is interesting to note that also in model I (with a 130-fs equilibration between C671 and C681 and a 3-ps intrinsic charge separation rate) the dominant phase at 270 K in charge separation has a rate of tens of picoseconds, which is in agreement with most results obtained so far. It is conceivable that in reality both fast and slow energy transfer processes coexist in RCs of PS II as is also observed in the major plant light harvesting complex LHC II (Kwa et al., 1992b; M. Du et al., personal communication). Low-temperature experiments with high time resolution are needed to decide in this matter.

The third and fourth eigenvalues are almost independent of the choice of fast or slow equilibration. These eigenvalues are determined by the distribution of the free energy difference between the charge-separated and singlet-excited state of P680 and by the parameters that describe C681. The eigenvalues coincide globally with the results of nanosecond transient absorption and fluorescence experiments by a number of groups (Booth et al., 1990, 1991; Roelofs et al., 1991, 1993; Volk et al., 1993; Hastings et al., 1992). The models agree in particular with experimental results (Booth et al., 1991; Roelofs et al., 1993), in that they predict several nanosecond decay phases at ambient temperatures (77 and 150 K) that become shorter lived upon raising the temperature. The strong 100–200-ps component at higher temperatures observed by Roelofs et al. (1991), Hastings et al. (1992), and Gatzen et al. (1992), is now suggested to arise from the decay of C681 via C671. The strong temperature dependence and large contribution to the ingrowth of the radical pair of this component provides a good test for the model in future experiments.

The models also predict a 2–6-ns component at all temperatures and thus suggest that such a decay component does not necessarily arise from uncoupled chlorophyll, in agreement with suggestions in Roelofs et al. (1993). At 10 K the 4.4-ns component does not arise from recombination, but reflects the lifetime of excitations trapped in C681. At high temperatures, however, the 2–6-ns components originate from charge recombination.

Heterogeneity and inhomogeneity

The temperature dependence of fluorescence and triplet quantum yields described in this contribution suggests that charge separation in reaction centers of PS II has to be described by at least three rather different ΔG values. The data may be explained by three conformational substates of the radical pair, caused by structural or dynamical heterogeneity. However, it is also possible that the 3 ΔG values reflect a broad distribution of ΔGs centered around 50 meV. A broad, inhomogeneous distribution of ΔG may be caused by various structural differences in the environment of P680 and the Pheo acceptor such as hydrogen bonds and side-chain arrangements. Also, variations in the nature of the P680 donor (coupling strength, monomeric/dimeric/trimeric nature, Kwa et al., 1994b) may have a direct influence on ΔG. The width of the distribution of $\sim$60 meV seems large in comparison to the average value. However, it is less than four times larger than the inhomogeneous width of P680*, which we estimate to be about 16 meV.

A heterogeneous distribution of the free energy difference will relate the three ΔG values from the simulation to three discrete energy gaps. The heterogeneity may be dynamic, in which case the initially formed radical pair state will relax to states characterized by a larger ΔG. This is caused by conformational rearrangements of the protein upon radical pair formation, and a "relaxed radical pair" will be formed (see Woodbury and Parson, 1984 and references therein; Schlodder and Brettel, 1988). In this case the smaller ΔG can only be important for the equilibrium between the singlet excited and radical pair states if relaxation to states with a larger ΔG is slower than the backward process. The heterogeneity may also be structural, in which case it may relate to recently reported heterogeneity in the P680 Q_y absorption spectrum (Van Kan et al., 1990, Van der Vos et al., 1992, Otte et al., 1992, Kwa et al., 1994b). We note, however, that the present data do not allow us to distinguish between inhomogeneity and structural or dynamical heterogeneity.

The distributions of ΔG and radical pair lifetimes will have experimental consequences. The most obvious one is that recombination in centers with a small ΔG will contribute more to the fluorescence than in those with a large ΔG. This explains why in general recombination lifetimes appear shorter in fluorescence than in transient absorption. On the other hand, when only the longest recombination lifetimes are "selected" by neglecting 6–12-ns phases (by attributing them to uncoupled Chl), centers with a large ΔG will be selected. In our opinion, this is the reason why Volk et al., (1993) find a higher ΔG value ($\sim$120 meV). The finding that there is no considerable magnetic field effect on the 6–12-ns phase (Volk et al., 1993) could just be the result of the shorter radical pair lifetime, which causes singlet-triplet mixing to take place only to a minor extent in centers with a small ΔG.

Contrary to previous suggestions (Booth et al., 1990, 1991, Roelofs et al., 1991), it appears not to be necessary to assume a considerable temperature dependence of the free energy

difference. This suggests that charge separation is not accompanied by large entropy changes.

APPENDIX

The model we have used for the RC of PS II contains four distinguishable excited states denoted as: A = C671, B = C681, C = P680, and D = $P^{+}I^{-}$. This leads to the following set of coupled linear differential equations (see Fig. 8):

$$\frac{dA}{dt} = -(k_{1A} + k_1 + k_3)A(t) + k_2 B(t) + k_4 C(t) \tag{1}$$

$$\frac{dB}{dt} = -(k_{1B} + k_2 + k_5)B(t) + k_1 A(t) + k_6 C(t) \tag{2}$$

$$\frac{dC}{dt} = -(k_{1C} + k_4 + k_6 + k_f)C(t) + k_3 A(t) + k_5 B(t) + k_b D(t) \tag{3}$$

$$\frac{dD}{dt} = -(k_{1D} + k_b)D(t) + k_f C(t) \tag{4}$$

whereby:

$$k_{1X} = k_{RX} + k_{1SX} + k_{1CX},$$

for radiative decay, intersystem crossing and internal conversion;

$$X = A, B, C;$$

$$k_{1D} = k_{1D} + k_{1CD} f(T),$$

for triplet formation from the radical pair and internal conversion;

$$k_{1CX,D} = k_{1CX,D} \, (T = 0 \text{ K}) * f(\text{T})$$

(It was found in the CP47 antenna complex that the temperature dependence of the fluorescence and triplet yield is best described by assuming only for the internal conversion rate a dependence on temperature according to the energy gap law in the strong coupling limit (Groot et al., unpublished results).);

and $f(T)$ = normalized decay rate for a radiationless transition between two electronic states with an activation barrier E_A in the strong coupling limit of the energy gap law (Englman and Jortner, 1970).

$$f(T) = \frac{C}{(k_B T_{eff})^{1/2}} \exp(-E_A/k_B T_{eff}) \tag{5}$$

where the effective temperature is defined in the form

$$k_B T_{eff} = \frac{1}{2} \hbar\omega_m \coth(\beta\hbar\omega_m/2) \tag{6}$$

C is a normalization constant, $\beta = 1/k_B T$, ω_m the mean vibrational frequency and E_A the activation energy. For the last two parameters the values obtained from a fit of the temperature dependence of the fluorescence and triplet quantum yield of the CP47 antenna complex to Eq. 5 are used, i.e., $\omega_m = 350 \text{ cm}^{-1}$ and $E_A = 650 \text{ cm}^{-1}$ (Groot, unpublished). The equations for triplet formation via the radical pair, triplet formation from states B and C and fluorescence yield are given by Eqs. 7, 8, and 9 respectively:

$$\frac{d}{dt} TR_{RP} = k_{1D} D(t) \tag{7}$$

$$\frac{d}{dt} TR = k_{1SB} B(t) + k_{1SC} C(t) \tag{8}$$

$$\frac{d}{dt} FL = k_{RA} A(t) + k_{RB} B(t) + k_{RC} C(t) \tag{9}$$

The steady-state solutions (denoted A_∞, B_∞, C_∞ and D_∞) are found by solving Eqs. 10–13.

$$\frac{dA}{dt} = -\epsilon_A I(t) \tag{10}$$

$$\frac{dB}{dt} = -\epsilon_B I(t) \tag{11}$$

$$\frac{dC}{dt} = -\epsilon_C I(t) \tag{12}$$

$$\frac{dD}{dt} = 0 \tag{13}$$

where:

$\epsilon_{A...C}$ = excitation density or relative oscillator strength; and $I(t)$ = number of incident photons (i.e., average number/unit of time for the steady-state fluorescence measurements and for the flash-induced T–S measurements; this is the total number of photons of a 6-ns flash).

And so:

$$TR = k_{1D} \int_0^t D(\tau) \, d\tau = k_{1D} D_\infty \int_0^t I(\tau) \, d\tau \tag{14}$$

can be written for steady-state. From this follows that the triplet quantum yield of the radical pair mechanism is given by:

$$\Phi_{TR} = k_{1D} D_\infty \tag{15}$$

Similar expressions can be written for the C681 and P680 triplet yield and the total fluorescence yield:

$$\Phi_{TRantenna} = k_{1SB} B_\infty + k_{1SC} C_\infty \tag{16}$$

$$\Phi_{FL} = k_{RA} A_\infty + k_{RB} B_\infty + k_{RC} C_\infty \tag{17}$$

To obtain the kinetic solutions, Eqs. 1–4 are solved numerically under the boundary conditions (Eqs. 18–21) with the rates determined from the steady-state simulations.

$$A(0) = \epsilon_A \tag{18}$$

$$B(0) = \epsilon_B \tag{19}$$

$$C(0) = \epsilon_C \tag{20}$$

$$D(0) = 0 \tag{21}$$

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