Fluorescence Lifetime Spectrum of the Plant Photosystem II Core Complex: Photochemistry Does Not Induce Specific Reaction Center Quenching

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ABSTRACT: The photosystem II kinetic model (diffusion or trap-limited) is still much debated. There is discussion about whether energy transfer from the core antenna (CP47 and CP43) to the reaction center complex (D1−D2−cyt b_{559}) is rate-limiting (transfer to trap-limited). This study investigates this problem in isolated core particles by exploiting the different optical properties of the core antenna and the reaction center complex near 680 nm, due to P680 and an isoenergetic pheophytin. This was used as a marker feature for the reaction center complex. If the transfer to the trap-limited model were correct, assuming excited-state thermalization, the specific reaction center fluorescence decay lifetime should be shorter near 680 nm, where there is reaction center complex specificity, than at the other emission wavelengths. Such a selective reaction center feature was not observed in fluorescence decay measurements. At the experimental resolution used here, we conclude that the trap-limited energy transfer to the reaction center could, at the most, be 20% limiting. Thus, the transfer to the trap-limited model is not supported. A kinetic, compartmental analysis was also performed on the data, taking into account a large number of separate measurements and the associated errors. Target analysis, considering these intermeasurement errors, yielded two minima which adequately describe the fluorescence lifetime data. The nonunique nature of the description is due to the fact that we have taken into consideration these intermeasurement errors. In our case, due to these errors, a correct kinetic model interpretation required additional experimental information.

PSII of higher plants is a multisubunit, chlorophyll−carotenoid binding complex which catalyzes the conversion of light energy into chemical energy. Energy is absorbed by the pigment−protein antenna subunits (complexes) and is transferred, on a picosecond time scale, to the reaction center complex, which binds both the primary donor, P680, or, possibly, a reaction center accessory chlorophyll (*1, 2*) and the primary acceptor pheophytin. The primary donor−acceptor charge separation creates an electrochemical gradient of ∼1.6 eV. The thermodynamic efficiency of the photosystem is high, being on the order of 85−90%. Approximately 80% of the chlorophyll is bound to the four complexes constituting the so-called "external antenna", and the remaining 20% (35−40 chlorophylls) is bound to the three core subunits (CP43, CP47, and D1−D2−cyt b_{559}; e.g., ref *3*); CP43 and CP47 possess only antenna function, while the D1−D2−cyt b_{559} complex is the reaction center subunit. Intact PSII, embedded in thylakoid membranes, is usually reported to have an average fluorescence decay lifetime of 300−400 ps (e.g., refs *4*−*7*) which is dominated by primary photochemistry. The recent report of the very short average lifetime for detergent-purified PSII (*8*) of 150 ps is probably due to removal of antenna complexes during sample preparation. An important functional question concerning primary photosynthetic processes in PSII is an understanding of the processes which determine the 300−400 ps photochemical trapping time. This subject has been debated, often with passion, over the past 20−25 years, though different points of view still exist. Basically, three factors are involved: (i) antenna size due to multiple-site "degeneracy" (antenna effect), (ii) rate-limiting kinetic processes in the antenna [diffusion-limited (DL)],[1] and (iii) kinetic limitations only in the reaction center at the level of primary charge separation [trap-limited (TL)]. Significant agreement exists only for the antenna effect which is generally considered to be important; this was recently confirmed experimentally (*4*), and it was suggested that the external PSII antenna effect may account for 60−70% of the trapping time. On the other hand, there is little agreement about whether PSII is DL or purely TL. Interpretation of earlier time-resolved fluorescence data suggested to many people in the field that an essentially trap-limited situation exists (e.g., refs *9* and *10*), i.e., a situation in which very rapid antenna excited-state equilibration occurred (10−20 ps) and photochemical trapping proceeded from this equilibrated state. This point of view was, however, questioned (*3, 11*), and more recently, the suggestion that a kinetic antenna-based limitation exists has been supported by other laboratories (*8, 12*). Thus, the current idea is that PSII kinetics is a mixture of DL and TL.

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[1] Abbreviations: CRS, core ratio spectrum; DL, diffusion-limited; TCSPC, time-correlated single-photon counting; TL, trap-limited; TTTL, transfer to trap-limited.

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An interesting version of the DL mode is the so-called transfer to trap-limited (TTTL) case. In this model, energy transfer from CP43 and CP47 to the reaction center complex is considered to be rate-limiting. For PSII, the TTTL model was initially proposed on the basis of the crystallographic structure presented by Zouni et al. (*13*). In this structure, it is evident that the nearest-neighbor chlorophyll distances among CP43, CP47, and the reaction center pigments (bridge chlorophylls) are in the range of 14−17 Å, considerably greater than the average nearest-neighbor distances within each of the complexes (*13*). Detailed excited dynamics calculations based on this structure were subsequently presented by Vasil'ev et al. (*14*), who concluded that the reaction center complex was indeed somewhat energetically isolated from the internal antenna complexes and that this "distance barrier" leads to the TTTL model for the PSII core, and consequently also for PSII as a whole. It seemed therefore possible that the physical site of the DL component to PSII trapping dynamics, discussed above, could be due to the internal antenna−reaction center complex interface. The calculations of Vasil'ev et al. (*14*), based on the Foerster transfer mechanism, however are not without problems. Thus, while the crystallographic structure, on which they are based, provides reliable center to center distance data, the spectroscopic properties of the specific bridge chlorophylls are unknown. This is not a minor problem as the chlorophyll *a* spectral forms in the internal antenna complexes span an energy range of $\sim 2.5 k_B T$ at room temperature (*15*). Thus, the Foerster overlap integrals for donor−acceptor couples are expected to display considerable variability. In their overlap integral calculations, these authors used the Shipman values (*16*) for chlorophyll in solution, which are quite different from those for protein-bound chlorophylls (*17*) in which the antenna band shapes are considerably narrower, due to the characteristic electron−phonon coupling of protein-bound chlorophylls and, as a consequence, the overlap integrals are changed.

With regard to the dipole orientations, due to the relatively low (∼3.5 Å) resolution obtained for these PSII core structures, as well as the fact that the molecular mechanics models used to interpret the X-ray electronic density are optimized for the protein backbone and not specifically for the geometry of the cofactors, the estimates of the geometrical coordinates of the chlorophyll tetrapyrrole ring, on which the determination of the transition dipole orientations is based, are not very reliable. Furthermore, the refractive index for the antenna environment is pretty well unknown, and it may well differ within the protein with respect to the protein−protein interfaces. This point has been recently discussed (*18*). Thus, while the suggestion of Vasil'ev et al. (*14*) provided interesting and novel information, further, independent, examination of the question was necessary.

In a very recent publication, another structure-based kinetic model was presented (*19*) in which the more recent and slightly higher-resolution PSII core structure (*20*) was used and the authors once again supported the TTTL model. In their calculations, the authors used what are thought to be corrected transition dipole orientations with respect to the earlier structure. In addition, they attempted to overcome the problem of relating the site energies to the crystal structure. Their approach was to perform spectral curve fitting of absorption, linear dichroism, and circular dichroism spectra, mostly measured at 77 K, of the isolated core complexes (CP43, CP47, and D1−D2−cyt b_{559}) using a calculated model chlorophyll band shape. It is difficult to evaluate this band shape as the phonon and vibrational modes used are not stated and the calculated chlorophyll absorption band shape is also absent and not compared with experimental measurements. The assumption of some average value for the Huang−Rhys factor of each chlorophyll is a rough approximation and will lead to errors in band shapes. The fit descriptions for the steady-state spectra are not precise, and the isolated CP43 absorption spectrum is blue-shifted with respect to published spectra (see, for example, ref *15*). The structure-based calculations of the excitonic interactions were determined using a genetic algorithm, and these data were used to determine the site energies for the 35 core chlorophylls. There are however, inevitably, problems associated with this ambitious attempt, and these are underlined by the large differences in site energies calculated with respect to another recent attempt (*21*). The differences in the estimated chlorophyll *a* site energies range from a minimum of 0.8 nm for chlorophyll 49 [nomenclature according to Loll et al. (*20*)] to a maximum of 12.6 nm for chlorophyll 34. It is evident that, at present, there is no room to reach a reliable kinetic conclusion based on sophisticated model calculation due to the marked uncertainty of the input data.

This point is further underlined in the so-called three-compartment model of Figure 17, which summarizes the main conclusions of this study. One notices that the rate constants for energy transfer into the reaction center from CP43 and CP47 are 2−3 times greater than for energy flow out of the reaction center. This conclusion is surprising as the three complexes are known to be almost isoenergetic (*22*) and site "degeneracy" is 2−3 times greater for the antenna complexes than for the reaction center. This casts serious doubt on the conclusions.

We now turn to the experimental studies. Vasil'ev et al. (*23*) published a kinetic analysis using fluorescence decay measurements of the isolated His-tagged PSII core particle from *Synechococcus*, which was suggested to support the TTTL model already proposed by these authors. This analysis, however, contains several unusual features. First, the fluorescence decay, apparently under conditions of open reaction centers, possesses a 950 ps decay component, which has not been observed either previously or successively in photochemically active core particles (refs *24*−*27* and this paper). This suggests the presence of closed reaction centers in their sample. Second, as recently pointed out by Miloslavina et al. (*25*), the model analysis introduces seven charge separation intermediates, for which no experimental evidence was presented. They seem to be merely fitting parameters. Recently, the Holzwarth group (*25, 28*) examined the fluorescence decay also of a *Synechococcus* core preparation and additionally performed a careful transient absorption analysis, yielding very different results and a different model-based conclusion. The transient absorption analysis demonstrated the presence of several radical pair intermediates, with picosecond lifetimes, which were incorporated into the compartmental models elaborated in both the transient absorption (*28*) and fluorescence decay studies (*25*). It was concluded that energy transfer out of the core antenna complexes was in the 1−2 ps range and could not be rate-

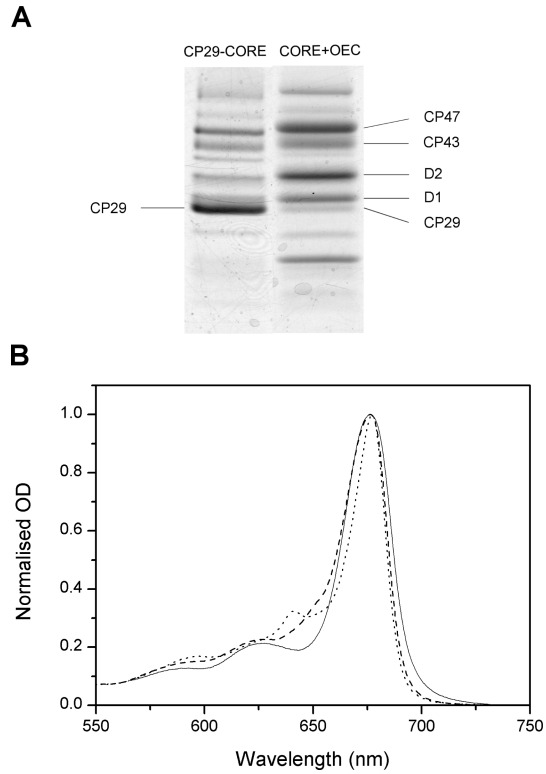

FIGURE 1: SDS−PAGE polypeptide composition of the PSII core preparation (A) and room-temperature absorption spectra of the core preparation and its component complexes (B). In panel A, the gel band identifications are on the figure. In panel B, the solid line is for the core preparation, the dashed line for the CP29−core preparation, and the dotted line for the native CP29 spectrum. Spectral normalization is to the maxima.

limiting, in contradiction to the work of Vasil'ev et al. (*14, 23*), and that the TTTL model was not effective in describing the PSII core decay. Thus, total disagreement still exists in the literature on this matter. In this paper, we re-examine specifically this question of whether the TTTL mode is correct for the PSII core. To this end, we used a novel experimental approach in which the spectral differences between the reaction center complex and the core antenna complexes were examined and exploited. It was shown that the reaction center complex (D1−D2−cyt b_{559}) has a detectable specific absorption difference near 680 nm with respect to the core antenna complexes. This characteristic was used as a marker feature for the reaction center complex, so if the TTTL model were correct, the specific reaction center fluorescence decay should be somewhat faster than that for emission wavelengths where this reaction center specificity is absent. Such a selective reaction center feature was not observed in our measurements, and at our experimental resolution, we conclude that the energy transfer to the reaction center could, at the most, be ~10−20% limiting. Thus, the TTTL model is not supported.

MATERIALS AND METHODS

The PSII core from maize plants was prepared by the procedure of Ghanotakis et al. (*29*). The preparation had a chlorophyll *a*/chlorophyll *b* ratio greater than 15, and the polypeptide composition was confirmed by SDS−PAGE (Figure 1) using 15% polyacrylamide gels in 6 M urea [Laemmli discontinuous buffer system (*30*)]. Samples, equivalent to 1.5 μg of chlorophyll, were loaded into the wells in which the characteristic presence of the CP43 and CP47 bands and the D1−D2 bands are evident. A minor band running with an electrophoretic mobility characteristic of CP29 is also indicated in Figure 1. To understand whether this is spectroscopically significant, we have compared the room-temperature absorption spectra of the core preparation with that of a CP29−core preparation where the chlorophyll *a*/chlorophyll *b* composition and the chlorophyll *a* band widths indicate that the CP29/core ratio is approximately 1 (Figure 1). The presence of the chlorophyll *b* bands characteristic of CP29 at 640 and 650 nm is evident in the CP29−core preparation. On the other hand, in the core preparation we can see no evidence of these chlorophyll *b* bands. We therefore conclude that if CP29 is in fact a contaminant of our core preparation it is present at levels which are spectroscopically insignificant.

Preparation of the component chlorophyll−protein complexes of the PSII core (CP43, CP47, and D1−D2−cyt b_{559}) and determination of their absorption spectra have been described previously (*11, 15*). As the number of counts for each measurement was $>10^5$ at all wavelengths, the statistical errors in these absorption measurements are vanishingly small and fall within the spectral curves.

To determine the overall photochemical rate, time-resolved (picosecond) fluorescence decay measurements of the PSII core with open reaction centers (F_0) were performed in a 50 mM MES buffer (pH 6.0) containing 0.4 M sucrose, 5 mM $CaCl_2$, 10 mM NaCl, and the electron acceptor potassium ferricyanide (0.65 mM) using the time-correlated single-photon counting technique (TCSPC) with excitation at 632 nm. The excitation source was a pulsed diode laser (PicoQuant GmbH, Berlin, Germany), peaking at 632 nm, controlled by a controller PicoQuant PDL 800-B and operating at a repetition rate of 20 MHz. We estimate that under these conditions the sample absorption flux is approximately 14×10^{13} photons/s which gives a mean value of approximately 2×10^{-3} photons absorbed per millisecond for each core complex. Thus, triplet accumulation is not expected, and sample reoxidation is assured. The emission decays were collected through a monochromator (Jasco CT-10, Japan) at 10 nm intervals in the wavelength range between 670 and 710 nm and detected by a microchannel plate photomultiplier tube (Hamamatsu R3809U-51). Samples were changed every 30 min, in which time the sample was stable (not shown). The decay parameters, time decay constants and amplitudes, were obtained by an iterative reconvolution between a sum of weighted exponential decay functions and a response function (IRF). Moreover, the background (dark) intensity and the offset of the time scale are parameters in the decay function definition. The resulting curve is fitted to the experimental data by minimizing the χ^2 with variation in the set of parameters. The IRF was obtained by measuring the fluorescence decay of a fluorochrome having a single known lifetime (e.g., ref *31*). The use of a fluorochrome to measure the IRF adds a new parameter (the fluorochrome lifetime) to the fit function but has several advantages with respect to the more conventional use of a scattering medium. A suitable choice of the fluorophore permits, in fact, measurement of the IRF in the same energy region where the sample decay is measured, removing the possibility of effects of color on detection. Moreover, it is

possible to measure the IRF at the same count rate as the sample, a condition difficult to obtain using a scattering medium. In this way, the count rate during the measure is determined by the sample fluorescence and not by the scattering sample with a gain of accumulated counts. The fluorochrome was DCI′ (Exciton, Inc., Dayton, OH) dissolved in ethanol, having a characteristic lifetime of ~20 ps (*32*). Its lifetime was directly estimated by the fluorescence decay measurement and the value used in the fitting process. The minimization procedure has been performed with a modified version of the algorithm previously developed in the laboratory to use the measured decay of a known fluorochrome as the source of IRF. The software is based on the Minuit library from CERN, distributed with a C-fortran interface as "C-minuit". Both the "C-minuit" and our Minuit-based algorithm are freely available as open-source software. The algorithm allows not only the minimization but also error analysis and determination of χ^2. After a number of tests, we find that the effective time resolution under our experimental conditions is better than 10 ps. The overall photochemical rate was then determined from the relevant DAS components, obtained by the fit analysis, as the $\tau_{av} = \sum A_i\tau_i/\sum A_i$ values at the various experimental wavelengths. The τ_{av} has been previously shown (*4*) to represent the correct determination of the overall photochemical rate, distinct from the mean lifetime value ($\tau_m = \sum A_i\tau_i^2/\sum A_i\tau_i$), which is sometimes used and can be directly obtained from the overall decay measurements.

Target compartmental modeling of the experimental data was performed using a Matlab-derived model. Model compartments were CP43, CP47, and D1−D2−cyt b_{559} plus two charge separation intermediates, following Miloslavina et al. (*25*). The population-weighted Boltzmann factors on which the rate constants among the three chlorophyll protein complexes were derived are from our earlier studies on the equilibrium excited-state population in each of these complexes (*15, 22, 33*).

RESULTS AND DISCUSSION

While the principle chlorophyll−protein complexes making up PSII are approximately isoenergetic (*15, 22*), absorption and fluorescence spectral differences are apparent in the isolated complexes. This is because the reaction center complex is optically intense in the 680 nm region (Figure 2) due to the presence of P680 itself, in addition to one of the two bound pheophytin molecules (*33, 34*). These spectral differences engender absorption and fluorescence selectivity at specific wavelengths in the 680 nm region, and these differences may, in principle, be exploited in probing details of photosystem function. A pertinent example of this is our earlier fluorescence quenching study of PSII particles (*11*) in which an emission selectivity of the reaction center complex (D1−D2−cyt b_{559}) in the 670−690 nm region enabled identification of specific reaction center fluorescence quenching during primary photochemistry. On the basis of this, it was suggested that kinetically limiting processes are present in the antenna and that PSII must be considered a mixture of TL and DL, a conclusion subsequently confirmed by others using different experimental approaches (*8, 12*). To investigate whether an important DL site is associated with kinetically slow transfer from CP43 and CP47 to the reaction center complex (TTTL), we now use an approach which is in principle similar to the previous one (*11*).

The initial part of the analysis begins from the room-temperature absorption spectra of the three core pigment−protein complexes (CP43, CP47, and D1−D2−cyt b_{559}), in the Q_y region. In Figure 2, the optical absorption differences between the reaction center complex and the sum of the core antenna complexes can be seen. It is evident that the reaction center complex is somewhat enriched in absorption transitions near 680 nm. A clear way to illustrate these differences is shown in Figure 2, where the so-called core ratio spectrum (CRS) is presented. This is simply the ratio of the linear sum of the absorption spectra of the complexes (eq 1), where the italic letters indicate the appropriate weighting factors (see the figure legend) derived from crystallographic structure stoichiometries (*13*)

$$\mathrm{CRS_A}(\lambda) = \frac{a\mathrm{CP47}(\lambda) + b\mathrm{CP43}(\lambda)}{a\mathrm{CP47}(\lambda) + b\mathrm{CP43}(\lambda) + c\mathrm{D1{-}D2{-}cyt}\ b_{559}(\lambda)} \quad (1)$$

As expected, the $\mathrm{CRS_A}(\lambda)$ displays a pronounced "hole" with its minimum close to 680 nm. As mentioned above, this is due to P680 and possibly also to one of the two pheophytins which is almost isoenergetic with P680 in the isolated complex (*34, 35*). It has recently been suggested that this pheophytin is somewhat blue-shifted in the isolated complex with respect to its wavelength position in vivo (*36*), in measurements performed at 1.7 K. We do not know whether this conclusion is also relevant to the long-wavelength pheophytin with respect to the isolated core complex at room temperature. On the other hand, CP43 and CP47 do not possess distinct absorption spectral forms, or states, at this wavelength (*15*). Due to the large interpigment distances among these three complexes (*13*), chlorophyll−chlorophyll interactions are weak and the denominator of eq 1 therefore represents the absorption spectrum of the core complex.

The $\mathrm{CRS_A}(\lambda)$ of Figure 2 refers to the absorption spectra. However, in the case of excited-state equilibration, this ratio spectrum applies exactly also to the fluorescence emission spectra via the Stepanov equation (*37*), as shown here (eq 2)

$$\frac{F(\nu)}{A(\nu)} = D(T)\nu^2 e^{-h\nu/k_B T}$$
$$\frac{A_{43+47}(\nu)}{A_{43+47+\mathrm{D1D2}}(\nu)} = \frac{F_{43+47}(\nu)}{F_{43+47+\mathrm{D1D2}}(\nu)} \quad (2)$$

where $F(\nu)$ and $A(\nu)$ are the fluorescence and absorption spectra, respectively, and the subscripts specify the complexes involved, $D(T)$ is a temperature-dependent function, independent of frequency, ν is the frequency, h is Planck's constant, and k_B is Boltzmann's constant. Thus, Figure 2 also represents the core ratio spectrum in emission [$\mathrm{CRS_F}(\nu)$], as described in eqs 1 and 2 under the assumption that each complex has a similar fluorescence yield. We now consider the case in which the fluorescence yield differs between complexes and, in particular, the case in which the overall photochemical kinetics are limited by slow energy transfer from CP43 and CP47 to the reaction center complex (TTTL model). In this case, we expect that the fluorescence yield of the D1−D2−cyt b_{559} complex will be decreased with respect to CP43 and CP47 due to this kinetic bottleneck and

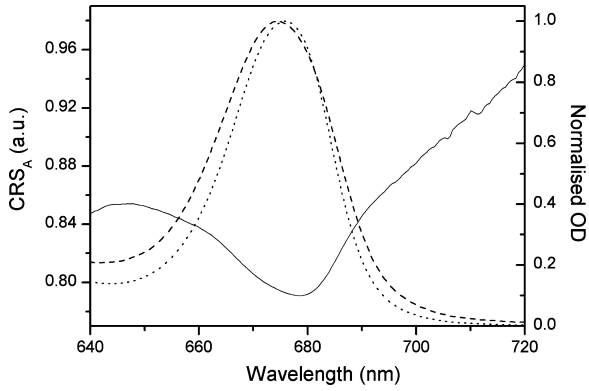

FIGURE 2: Absorption spectra of the PSII core antenna and reaction center complex (dashed curve and dotted curve, respectively) and the absorption core ratio spectrum [$CRS_A(\lambda)$] determined according to eq 1 and using the pigment binding stoichiometries derived from the crystallographic structure (*13*) as weighting factors for the complexes (solid curve).

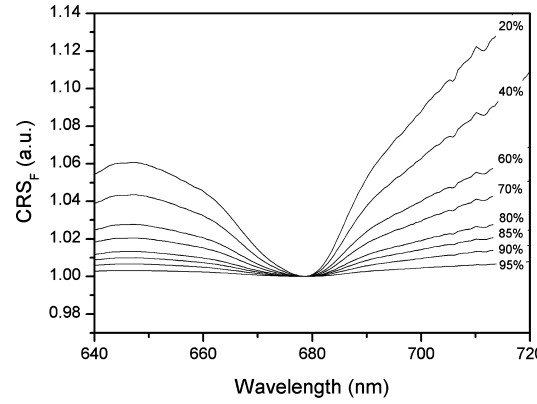

FIGURE 3: Core ratio spectrum [$CRS_F(\nu)$] determined using eq 3, for different values of the weighting factor (c'), for the D1−D2−cyt b_{559} complex. All $CRS_F(\nu)$ values are arbitrarily normalized at their minima, near 680 nm. The percent numbers given in the figure are the relative fluorescence yields of the reaction center complex, obtained using different values of c'.

fast primary photochemistry. This situation may be represented by the Ketskemety et al. (*38*) modification of the Stepanov expression (eq 3)

$$\frac{F(\nu)}{A(\nu)} = D(T)\nu^2\Phi(\nu)e^{-h\nu/k_BT} \quad (3)$$

where $\Phi(\nu)$ is the fluorescence yield across the core complex absorption band. This function modulates the equilibrium excited-state distribution considered by Stepanov (see above). In the case of the TTTL model situation, where photochemical-induced quenching is localized on the reaction center complex, $\Phi(\nu)$ will decrease in the spectral region where the D1−D2−cyt b_{559} complex absorption is specifically more intense. This situation will be called $\Phi_Q(\nu)$. From eqs 2 and 3, and noting that the $A(\nu)$ terms offset as reaction center fluorescence quenching does not influence the absorption spectrum, we can write the following equivalences

$$\frac{F_Q(\nu)}{F(\nu)} = \frac{\Phi_Q(\nu)}{\Phi(\nu)} = \frac{a\text{CP47}(\nu) + b\text{CP43}(\nu) + c'\text{D1/D2}(\nu)}{a\text{CP47}(\nu) + b\text{CP43}(\nu) + c\text{D1/D2}(\nu)} = CRS_F(\nu) \quad (4)$$

where the subscript Q indicates the photochemically quenched core complex. This situation, via the Stepanov equations (eqs 2 and 3), can be written in terms of the core complex absorption ratio (eq 4), similar to eq 1 and using the same weighting factors, except for the D1/D2(ν) weighting factor $c' < c$. Thus, the $CRS_F(\nu)$ expression of eq 4 represents the fluorescence yield ratio in the presence of specific reaction center quenching ($c' < c$). By inspection of the CRS expressions in eqs 1 and 4, it is evident that if the TTTL model is correct then the $CRS_F(\nu)$ spectrum of eq 4 is expected to display, to some extent which is to be defined, the 680 nm "hole" structure present in Figure 2. On the other hand, if energy transfer to the reaction center in the PSII core is not at all kinetically limiting, the $CRS_F(\nu)$ spectrum is expected to be substantially constant. This situation is represented graphically in Figure 3 for different values of c'.

The fluorescence yield ratio in eq 4 may also be written as

$$\frac{\Phi_Q(\nu)}{\Phi(\nu)} = \frac{k_F\tau_{avQ}(\nu)}{k_F\tau_{av}(\nu)} \quad (5)$$

As $\tau_{av}(\nu)$ is taken to be constant for the equilibrated case, the ratio $\Phi_Q(\nu)/\Phi(\nu)$ is linear with $\tau_{avQ}(\nu)$. Thus, the $CRS_F(\nu)$ spectrum of Figure 3 also represents the variation of $\tau_{avQ}(\nu)$ across the PSII core absorption fluorescence band.

The relation between the fluorescence yield of the reaction center complex and the rate of photochemistry for the TL and the TTTL model systems is now examined. To this end, we employ a compartmental type kinetic model of the PS II core, of the type recently published by Holzwarth and coworkers (*25*) which provides a satisfactory description of our experimental data. The experimental and analytical details leading to this model will be discussed below. We present the model at this stage to examine the primary photochemical rate with respect to the reaction center fluorescence yield. This model is similar to that of Miloslavina et al. (*25*) in that it includes two charge separation relaxation intermediates and also energy transfer from the antenna complexes is fast; i.e., it is not of the TTTL type. However, we point out that our conclusions are general and not model-dependent. They apply equally to even strongly TTTL type situations (unpublished observation). Our analytical approach is similar to that of Miloslavina et al. (*25*), with which they examined the average photochemical fluorescence lifetime (τ_{av}) as a function of the rate of energy transfer from the antenna complexes to the reaction center complex. In Figure 4, the rate of energy transfer from the two antenna complexes is rescaled by different amounts (scaling factor in the figure) and both the primary photochemical rate (τ_{av}^{-1}) and the fluorescence yield of the reaction center complex are determined. In Figure 4, we present both the τ_{av} and the fluorescence yield of the reaction center complex versus the rate of energy transfer to the reaction center complex. As previously discussed by Miloslavina et al. (*25*), the τ_{av} plot clearly shows the transition between the purely TL and a system in which there is some TTTL contribution. This transition is equally evident in the fluorescence yield plot of the reaction center complex which scales linearly with τ_{av}^{-1}. We stress that this latter conclusion is not model-dependent, and the two parameters may be considered as providing equivalent information. Thus, in the TL model situation, both the fluorescence yield [Φ or $\Phi(\nu)$] and τ_{av} are insensitive to rates of energy transfer into the reaction center complex, while in the presence of some TTTL model contribution,

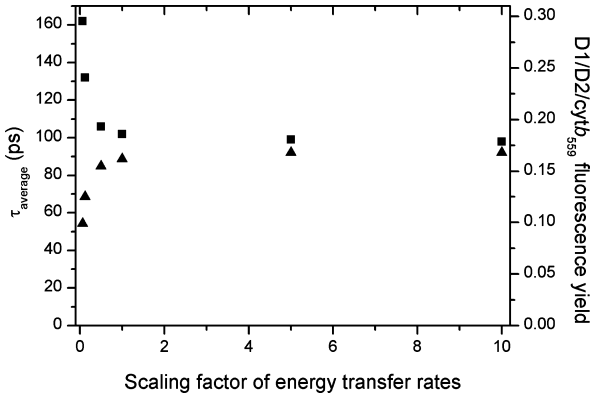

FIGURE 4: Dependence of the excited-state average lifetime [τ_{av} (■)] and the relative fluorescence yield of the reaction center complex (▲) on the scaling of energy transfer rates from the antenna complexes to the reaction center complex. Calculations were performed with the kinetic model of Figure 8B which represents the unity scaling factor. The other scaling factor values indicate the values by which the rates of energy transfer into the reaction center complex were multiplied.

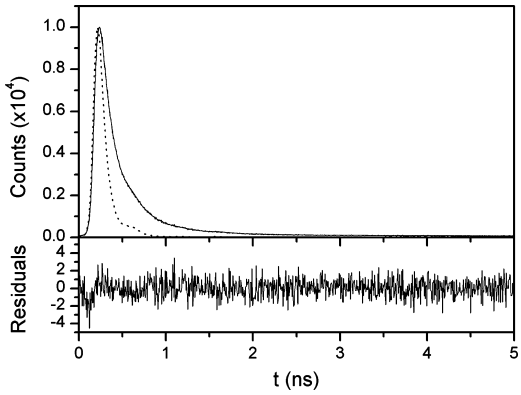

FIGURE 5: Typical fluorescence decay measurement of the maize PSII core at the F_0 level of fluorescence (noisy curve). The four-exponential component fit (solid curve) and the instrument response (dotted curve) are also presented. The fit residuals are shown at the bottom.

they are not. As one can see in Figure 4, the reaction center complex becomes quenched [Φ_Q or $\Phi_Q(\nu)$], and this leads to an equivalent lengthening of τ_{av}. Thus, the $CRS_F(\nu)$ spectrum of Figure 3, which according to eq 5 also represents the variation of τ_{av} across the PSII core absorption fluorescence band, may be used to distinguish between the TL and TTTL models. It is obvious that the real situation could well be some combination of both modes. We propose to use the operative definition of 50%. That is, if it is more than 50% TTTL, we call it substantially TTTL. If, on the other hand, the TTTL contribution is less than 50%, we call it substantially TL.

To investigate the trapping model for the PSII core in terms of the rationale described above, two different, but theoretically similar, experimental approaches are possible. The most straightforward is to determine the steady-state fluorescence yield across the absorption fluorescence band, under conditions of active photochemistry, and to compare the data with the $CRS_F(\nu)$ spectra. This approach, however, suffers from the defect that, due to the very short fluorescence lifetime of the PSII core (Figure 5), the presence of even a tiny number of chlorophyll molecules which are not energetically coupled to the reaction center, and which therefore have a nanosecond lifetime, will lead to spectral distortion.

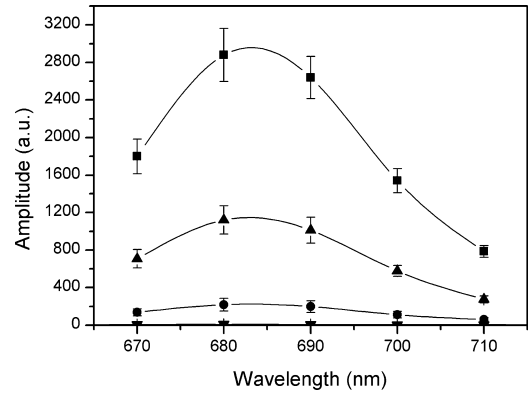

FIGURE 6: Decay-associated spectra for the maize PSII core preparation: (■) 46 ± 4 ps, (▲) 175 ± 13 ps, (●) 523 ± 42 ps, and (▼) 3112 ± 355 ps. Errors are the standard deviations determined from 12 separate measurements.

We have therefore adopted the rather laborious approach of determining the spectral dependence of τ_{av} by means of fluorescence decay measurements. In this way, the problem of uncoupled pigments is overcome as unwanted long lifetime components may be eliminated from the determination of τ_{av}.

Fluorescence decay experiments with the maize PSII core with open reaction centers were performed to determine τ_{av} between 670 and 710 nm. The signal-to-noise ratio for each measurement was good (10000 counts accumulated in the peak channel), and the data were analyzed globally to produce-decay associated spectra (DAS). Four-exponential decays were found to provide a satisfactory fit with χ^2 values of ~1.07. A plot of the residuals for a single measurement is presented in Figure 5. As we have previously discussed (*4*), the single-decay measurements display intermeasurement variation, and to achieve a satisfactorily low error distribution, we performed 12 separate measurements. The DAS are presented in Figure 6 as the mean amplitudes together with the relative standard deviations for each measurement wavelength. The dominant amplitude decay has a lifetime of 46 ± 4 ps followed by those at 175 ± 12, 523 ± 42, and 3100 ± 355 ps. The latter component, which has a very small amplitude (≈0.2%), is considered to represent uncoupled pigments.

While a detailed DAS analysis of the plant PSII core is not the main aim of this study, we notice that there are similarities and differences in the description of Figure 6 with respect to previous work of this kind on PSII core particle. In all cases, these were prepared from *Synechococcus*. The isolated plant core complex has not been previously examined. In our case, the dominant 46 ± 4 ps decay is probably equivalent to the 60−80 ps decay previously reported in *Synechococcus* (*23, 27*) and the recently published 41 ps component (*25*). The 523 ± 42 ps decay is presumably equivalent to the slowly decaying component variously described in the 300−500 ps range (*23, 25, 27*). The 175 ± 12 ps decay does not seem to have a clear equivalent in earlier cyanobacterial papers (*23, 27*), though Miloslavina et al. (*25*) have recently reported a 105 ps component. In this respect, we note that decay times very similar to those reported here were reported by Engelmann et al. (*4*) for PSII from thylakoids of the Chlorina mutant, from which the outer antenna complexes are almost entirely missing and which is therefore expected to resemble the

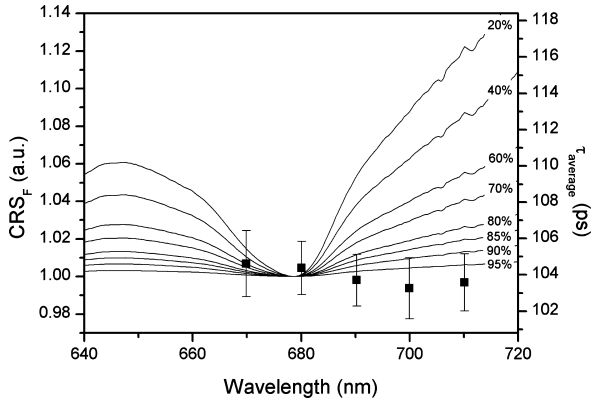

FIGURE 7: Distribution of the τ_{av} values at the various experimental wavelengths overlaid with the normalized $CRS_F(\nu)$ spectra taken from Figure 3. The error bars represent the interval mean estimate at the 95% confidence level. The means of the five τ_{av} data points are equalized at the minima of the $CRS_F(\nu)$, at 680 nm, equal to $\tau_{av} = 104$ ps.

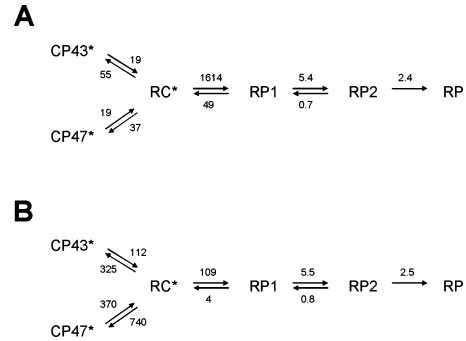

FIGURE 8: Compartmental kinetic models which provide almost identical DAS descriptions of the maize PSII core fluorescence decay. Rate constants, given in the figure, are given in inverse nanoseconds. Boltzmann factors were derived from the experimentally determined mean energy levels of these complexes (*3, 15, 33*), and the weighting is based on the crystallographic spectroscopic data. Other fit parameters were the three-component DAS description (Figure 6) and the τ_{av} value. Model A is of the TTTL type (lifetimes of 46 and 52 ps with a very low amplitude and 175 and 523 ps with a τ_{av} of 104 ps), while model B is of the TL type (lifetimes of 46, 179, and 524 ps with a τ_{av} of 104 ps).

purified plant core complex. Thus, the possibility that the 175 ± 12 ps decay may indicate a kinetic, and therefore possibly a structural, difference between the plant and cyanobacterial core complexes exists.

A significant difference with respect to the recent analysis of Miloslavina et al. (*25*) is our inability to detect the approximately 9 ps decay reported by these authors. This is at the very lowest edge of our instrumental resolution, while the resolution of the experimental setup of Miloslavina et al. (*25*) is somewhat greater than ours. However, our setup successfully resolved 11 ± 1 and 30 ps components in previously published multicomponent photosystem I fluorescence decay experiments (*39*), so we feel that a 9 ps decay should be detectable and resolved from the 46 ps one in this study. We have therefore attempted to analyze our data by including a fifth, fast, exponential process. In this way, an 8−9 ps process is in fact produced, but its amplitudes are negative at all wavelengths. Such a component would appear to be without physical sense in our case and at any rate is not equivalent to the positive amplitude 9 ps component of Miloslavina et al. (*25*). Thus, we are unable to confirm the presence of this decay using the plant PSII core particle.

With their improved experimental resolution, Miloslavina et al. (*25*) describe an approximately 2 ps component which has only negative DAS amplitudes. This component, and the undoubted presence of even faster transfer components, are clearly well below our experimental resolution. They are not, however, expected to modify our DAS amplitudes due to the rapidity of their decay that renders their contribution to the DAS negligible.

We now consider the distribution of the τ_{av} values at the various experimental wavelengths, which, of course, is the main point of this study. These have been calculated in the usual way as $\sum A_i\tau_i/\sum A_i$, where the i values are the 46, 175, and 523 ps decay components for each measurement at each wavelength (see Materials and Methods). Subsequently, the mean values with the relative errors were determined, and the data are presented in Figure 7 where they are normalized to and overlaid with the $CRS_F(\nu)$ spectra. The error bars represent the interval mean estimates at the 95% confidence level. It is apparent that for the five data points between 670 and 710 nm the τ_{av} values are substantially constant, lying in the range of 104.8−103.2 ps and the 95% confidence level mean estimates are between ±1.3 and 1.8 ps. It is therefore evident that, within the experimental resolution, these five points are indistinguishable and do not exhibit the spectral structure apparent in the $CRS_F(\nu)$ spectra. In other words, minimal values for τ_{av} near 680 nm (hole structure) are not apparent, in contradiction with the TTTL model. However, it is important to understand the limits of our resolution. This is achieved by normalizing the mean of the five τ_{av} data points to the minima of the $CRS_F(\nu)$, at 680 nm, equal to $\tau_{av} = 104$ ps. This normalization indicates that the upper values for the three mean interval estimates at wavelengths greater than 680 nm fall between the 5 and 15% $CSR_F(\nu)$ spectral curves and therefore suggests that our resolution may be considered to be on the order of 15%. Thus, we are led to conclude, on the basis of this experiment, that if energy transfer from CP43 and CP47 to the reaction center complex is limiting for the overall photochemical rate, as suggested in the TTTL model, this limitation must be rather small. It is certainly much less than 50% and probably even less than 15% of the overall rate. Thus, we conclude that the PSII core is substantially TL.

It will be noticed that the τ_{av} values determined here for the PSII core are close to 100 ps. This is in contrast to the relatively slow value initially published for the *Synechococcus* core in the earlier literature of 160 ps (*23, 27*) and the very fast value recently published for *Synechococcus* by the same laboratory (*25*) of ~60 ps. It is not very different from the approximate value of 120 ps of Vasil'ev et al. (*23*). The reasons for these variations are not clear. In the work of Miloslavina et al. (*25*), they suggested that their very fast value may be associated with a superior instrumental time resolution, as they were able to detect a decay component at 9 ps, which had not been previously noted. However, we point out that the 9 ps decay of Miloslavina et al. (*25*) is a minor decay which, if omitted, increases their τ_{av} value from 60 to 70 ps, and the discrepancy remains. In the papers from the Holzwarth laboratory (*25, 27*) and in the study by Vasil'ev et al. (*23*), a flow cell is used to help maintain the PSII centers in an oxidized state, and this is not the case in our analysis. However, our light intensity is extremely low (approximately 2×10^{-3} photons absorbed per millisecond

for each core complex) which assures reaction center oxidation by ferricyanide between flashes. In addition, we point out that in these measurements there are no long-lived decay components, characteristic of closed centers, though the study of Vasil'ev et al. (*23, 27*) does in fact contain a 950 ps decay, unusually long for open centers. Also, we point out the fact that the so-called free pigment component, in our case with a τ of ~3 ns, is unlikely to hide significant amounts of closed PSII traps as its amplitude is around 0.2%. Thus, we conclude that significant amounts of closed PSII centers are absent in our measurements. Of course, it should be mentioned that the fast Miloslavina et al. (*25*) sample was prepared by a completely different biochemical procedure with respect to other *Synechococcus* preparations and our plant preparation. As pointed out above, we cannot exclude the possibility that there are cyanobacterial−plant core differences.

In the final section of our paper, we report on a simple compartmental model which provides a satisfactory explanation of our open trap core decay data. While this is not the main point of our study, it is necessary, if our conclusion on basically TL kinetics is correct, that the decay data be described well by a TL model. Several words of caution are necessary here as the intermeasurement errors which are demonstrated in this paper are such that point numerical solutions make little sense, when interpreted in detail. The model inevitably has the imprecision inherent in the data which should be considered. In our case, these errors lead to nonunique fit minima. These general points should be taken into account when models, based on time-resolved data, are developed, and this is not usually done. Thus, exact numerical kinetic and thermodynamic parameters derived from modeling of time-resolved data should be treated with caution. With this in mind, we have sought a compartmental model description of the data presented in Figures 6 and 7 using a target modeling approach that was, to a large extent, based on the strategy of Miloslavina et al. (*25*), as these authors included two relaxation intermediates for primary charge separation for which experimental evidence, as pointed out above, exists. The multi-intermediate approach of ref *23* has no experimental support. For energy transfer between the three core complexes, we have used Boltzmann factors which come from our earlier studies of the mean energy levels of these complexes (*3, 15, 33*). Other fit parameters were the three-component DAS description (Figure 6) and the τ_{av} value. In this way, by target analysis and with different initial conditions, it is evident that two fit minima are present as two almost identical DAS descriptions of the experimental data were found (Figure 8). These have very different kinetic and thermodynamic characteristics which clearly underlines the care which is required when using time-resolved data in detailed kinetic modeling and furthermore shows, as we have already pointed out (*4*), that other criteria may need to be considered. The model in Figure 8A has slow energy transfer from the antenna complexes to the reaction center and fast primary photochemistry (TTTL model), while that in Figure 8B has fast energy transfer to the reaction center and slower primary charge separation (TL model). From the time-resolved data alone, there is no way of distinguishing between them as both descriptions fall within the experimental errors. However, as we have previously concluded, on the basis of the experiments presented in Figure 7, that the TTTL model is substantially incorrect, we are able to choose between the two model descriptions of Figure 8 in favor of Figure 8B (TL model). We conclude that these model parameters provide a reasonable representation of the PSII core dynamics. The analysis suggests quite fast transfer from the core antenna complexes to the reaction center (time constants in the 3−9 ps range) with primary radical pair formation around 9 ps. This is the model which we have used for the TTTL calculations presented in Figure 4 and have previously mentioned above. It is interesting to note that the model describes an essentially TL model for the PSII core, which can be seen from the unity scaling factor for the curves in Figure 4, with the model characteristics suggesting an almost borderline situation; i.e., the model solution is very close to the kinetic dividing zone between the pure TL and the TL with a slight TTTL contribution. It is noteworthy that this is exactly the same conclusion proposed in Figure 6 of ref *25*, even though some differences in experimental detail between their data and ours exist. We may therefore conclude that our data, based mainly on the spectral dependency of τ_{av} (Figure 3) and supported by the compartmental modeling of Figure 8, are in favor of a substantially TL situation for the PSII core, in agreement with the Holzwarth group.

Further Discussion. These results for the PSII core have a bearing on the more general problem of whether excitation trapping in PSII (core plus external antenna complexes) is TL or DL. The TTTL model is a particular case of the DL mode. As indicated in the introductory section, this important aspect of the primary processes in photosynthesis has been discussed for many years, and our interpretation suggests that both DL and TL processes occur in PSII. In this context, our analysis on the PSII core, together with the recent conclusions of Miloslavina et al. (*25*), suggests that the location of the diffusion-limited trapping component is not associated with energy transfer within the PSII core. It is therefore expected to be associated with energy flow either within the external antenna or between the external antenna and the core. In this context, the suggestion that excitation equilibration in aggregates of LHCII is kinetically "slow" (*12*) is interesting, though, of course, the interfacial regions between artificial LHCII aggregates, used in this study, may well be different from those in the natural PSII supercomplex. In addition, the possibility that transfer from the external antenna to the core complexes may be "slow" is suggested by the PSII computer structural simulations of Nield and Barber (*40*). These indicate that nearest-neighbor chlorophyll distances between the complexes of the external antenna and CP43 and CP47 may be >20 Å. Thus, if "linker" chlorophylls, which up to now have not been detected in crystallographic studies, should not be present, this could be a DL site.

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