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Articles

Investigations on the Reaction Pattern of Photosystem II in Leaves from *Arabidopsis thaliana* by Time-Resolved Fluorometric Analysis†

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ABSTRACT: The transients of normalized fluorescence yield induced by an actinic laser flash in dark adapted leaves of *Arabidopsis thaliana* plants were measured with new equipment, that was developed as part of this work and permits the covarage of a wide time domain of 8 decades from 100 ns to 10 s. The raw data obtained were processed and analyzed within the framework of the "3-quencher" model with Q_A as photochemical and $P680^{+\bullet}$ and ^3Car as nonphotochemical quenchers. Comparative measurements with hydroxylamine treated PS II membrane fragments from spinach revealed that the widely used "dogma" of virtually identical efficiency of photochemical (Q_A) and nonphotochemical ($P680^{+\bullet}$) quenching has to be revised: the constant of the latter exceeds that of the former by a factor of about 2. As a consequence, the probability of recombination between $P680^{+\bullet}$ and Q_A^- and its kinetics have to be explicitly taken into account for the interpretation of flash induced fluorescence yield transients. The analysis of the experimental data within this extended "3-quencher" model reveals that a fully consistent description is achieved for the data gathered from measurements with intact leaves from wild type plants excited with actinic laser flashes of different energies (number of photons per flash and unit area). On the basis of these results it is shown that, in dark adapted leaves excited with a single laser flash, $P680^{+\bullet}$ is predominantly (about 80% of the total reaction) reduced by Y_Z via nanosecond kinetics and Q_A^- reoxidation is dominated by a kinetics of about 150 μs that are ascribed to PS II complexes with the Q_B site occupied by PQ. The excess of excited chlorophyll singlet states decays to a significant extent via the carotenoid "triplet valve" with transient population of ^3Car. The present data provide the basis for analyses of *A. thaliana* mutants with modified lipid content and composition. The results of these investigations are described in an accompanying report (Steffen, R., Kelly, A. A., Huyer, J., Dörmann, P., and Renger, G. (2005) Investigations on the reaction pattern of photosystem II in leaves from *Arabidopsis thaliana* wild type plants and mutants with genetically modified lipid content, *Biochemistry 44*, 3134−3142).

Photosynthesis is the most important process for the utilization of solar radiation as unique source of Gibbs energy for development and sustenance of living matter. The key steps of this process take place in pigment protein complexes that are anisotropically incorporated into lipid bilayer membranes of comparatively high protein content. As an essential consequence of this anisotropic arrangement electron transport processes are vectorial with respect to the membrane normal. Therefore the coupling of electron and proton transfer reactions gives rise to membrane energization,

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and the electrochemical potential difference thus formed can be used as driving force for endergonic processes like ATP synthesis or active transport. The membrane acts as a functional element for these energy transducing reactions whereby the lipids provide an "impermeable" barrier to dissipative decay of the electrochemical potential difference via "undesired" ion fluxes. In addition to this general essential barrier function of the bulk lipid phase in energy transducing membranes (not only in photosynthesis), lipids are also reported to exert specific interactions with protein complexes and are relevant for their optimal structural and functional properties. Illustrative examples of this mode of lipid action have been reported: (i) the role cardiolipin in cytochrome *c* oxidase of the respiratory chain (*2*), (ii) the importance of phosphatidylglycerol (PG)[1] and digalactosyldiacylglycerol (DGDG) for the structural organization of light harvesting complex II (LHC II) in green plants (*3*−*5*), (iii) the influence of monogalactosyldiacylglycerol (MGDG) on the activity of violaxanthin deepoxidase (*6*), (iv) the role of PG for the trimerization of PS I in cyanobacteria (*7*), and (v) the role of sulfoquinovosyldiacylglycerol (SQDG) and digalactosyldiacylglycerol (DGDG) for the oxygen evolving photosystem II (PS II) in cyanobacteria and plants (*8*−*16*).

Basically two different approaches can be used to elucidate the role of specific thylakoid lipids for the reaction pattern of functional complexes embedded in the membrane: (a) extraction of these complexes from the membrane and either reconstitution in a defined lipid environment or modification of the system by lipase treatment (*8*−*10*) and (b) modification of the lipid composition by genetic means and in vivo analyses of the respective mutants. Both approaches have pros and cons for mechanistic studies. The latter approach has the great advantage that information is obtained that reflects the in vivo situation in plants rather than properties of isolated complexes that are prone to interference by artifacts owing to secondary effects caused by the isolation procedure. The study of the reaction pattern of PS II in whole cells, however, bears the problem of limited methodology because many of the highly sophisticated spectroscopic techniques cannot be used. The most powerful tools to address this problem are fluorometric methods that are widely used for studies in plant physiology (*17*). Complementary methods that allow monitoring of light induced absorption changes in plants (e.g. P700 turnover) are also now available (*18*).

At room temperature chlorophyll fluorescence mainly originates from the antennae system of photosystem II (PS II) (*19*, *20*), and measurements of the decay kinetics with high time resolution are appropriate to study the trapping of photons via photochemical charge separation (see ref *21* and references therein). On the other hand, the transients of fluorescence quantum yield induced by actinic light reflect changes in the functional state of PS II itself and formation/decay of quenchers in the antennae system of PS II. In the present communication we are focusing on the potential of the latter technique.

For studies of light induced fluorescence yield changes basically two different types of methods are distinguished with respect to PS II excitation, depending on the mode of actinic illumination: (i) multiple and (ii) single turnover techniques. Among the former approach two actinic light sources are most widely used: CW light in the fluorescence induction method, originally developed by Kautsky and Hirsch (*22*), and saturating multiple turnover pulses in the light-doubling method introduced by Bradbury and Baker (*23*). Despite their widespread application in photosynthesis research, an unambiguous and straightforward data interpretation is still a matter of debate (*24*−*28*), owing to the underlying complex dependencies of the fluorescence signal on the mechanistic details of the photosynthetic apparatus. Under multiple turnover excitation conditions several processes are induced that change the state of the photosynthetic system from its initial equilibrium state (usually the dark adapted state) to a new nonequilibrium steady state that is characteristic for the applied light conditions.

An alternative to the multiple turnover methods is the excitation of the photosynthetic apparatus by a single turnover laser flash so that the induced fluorescence transients reflect the time course of a sequence of individual reactions that eventually lead to relaxation of the system into its original equilibrium state. Therefore, single turnover flash experiments are expected to permit a more straightforward data interpretation. However, apart from the more sophisticated technical setup that is required for a sufficiently high time resolution, the deconvolution of the experimental traces into molecular parameters of the system is still not a simple procedure because different quenching components with overlapping time regions contribute to the overall signal. Therefore data interpretation requires detailed knowledge on the nature and kinetics of the components that undergo state changes that affect the relative fluorescence quantum yield. First experiments resolving the fast fluorescence yield changes induced by actinic single turnover flashes have been performed in the 1970s and were applied to study specific PS II reactions (*29*−*39*). On the basis of these experiments, three components of PS II (including its antennae) are currently considered to influence the chlorophyll (Chl) fluorescence yield in single turnover flash experiments: (i) the photoactive pigment P680, (ii) the secondary acceptor of PS II (Q_A) and (iii) carotenoids (Car) mainly located in the antennae complexes (for a recent discussion see ref *39*).

The present study describes the development of equipment that permits simultaneous monitoring of laser flash induced fluorescence yield transients in the wide time domain of 100 ns to 10 s. Experimental data are presented that show for the first time that the essential assumption on virtually identical quenching efficiencies of $P680^{+\bullet}$ and Q_A, currently used for the interpretation of flash induced fluorescence yield transients within the "3-quencher" model, has to be revised. Furthermore, the theoretical basis of flash induced measurements of the fluorescence quantum yield is discussed and an analytical approach in terms of rate constants presented to describe the observed reaction kinetics. In a second communication (*1*) this approach is used for monitoring and interpretation of flash induced changes of the chlorophyll

[1] Abbreviations: Chl, chlorophyll; Car, carotenoid; PS II, photosystem II; P680, photoactive Chl of the reaction center of PS II; Q_A and Q_B, primary and secondary plastoquinones of PS II; WOC, water-oxidizing complex; Y_Z, tyrosine 161 of the PSII D1 polypeptide; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol; fwhm, full width at half-maximum; EET, electronically excited energy transfer; LHC, light harvesting complex; PAR, photosynthetic active radiation.

fluorescence quantum yield in mutants of *Arabidopsis thaliana* with a modified lipid content and composition.

MATERIALS AND METHODS

Plant Growth Conditions. A. thaliana wild type plants (ecotype Columbia-2 (Col-2)) were germinated in Petri dishes containing a solidified growth medium (MS salts, 1% [w/v] sucrose, 1% [w/v] agarose) for 2 weeks before transfer to soil (*40*). The light regime for all growth conditions was 16 h of light/8 h of dark at a light intensity of 150 μmol/(m^2 s). Plants were dark adapted before each measurement for 1 h.

Preparation of PS II Membrane Fragments from Spinach. Thylakoid membranes were isolated from spinach leaves as described in ref *41*. PS II membrane fragments were prepared from thylakoid preparations according to the standard method (*42*) with modifications outlined in ref *43*.

Measurements of Transient Absorption Changes. Flash-induced absorption changes at 830 nm with a microsecond time resolution were measured under repetitive laser flash excitation with a single beam flash photometer as described in ref *44*. Measurements were performed with PS II membrane fragments suspended at a concentration of 50 μg of Chl/mL at room temperature in a buffer containing 10 mM NaCl and 50 mM MES (pH 6.5). The samples were excited by 10 ns (fwhm) laser flashes at 532 nm from a frequency-doubled Nd:YAG laser at a repetition rate of 1 Hz.

Measurements of Transient Fluorescence Yield Changes. Laser flash induced transients of fluorescence yield were monitored in the time range from 100 ns to 10 s. The original setup as described in ref *39* was modified by extending the sweep time to about 10 s (see also the instrument description in the Supporting Information). Extension of the sweep time was achieved by means of a digital storage oscilloscope (Delta 9500A, Gould Nicolet) that provides a special memory segmentation mode. The major problem in combining the measurement of fast fluorescence rise kinetics in the nanosecond time domain as described in ref *39* with subsequent monitoring of the slower relaxation kinetics in the microsecond to second time domain with a digital storage oscilloscope (DSO) is the high sampling frequency of 100 MS/s (Mega Samples per second) that is necessary to resolve the fast fluorescence rise kinetics. Due to the limited storage capacity of the DSO memory (1 Mega Word per channel) the sweep time would be limited to 10 ms. This limitation was surpassed by using the memory segmentation mode of the Delta 9500A. This mode allows partitioning the memory into a number of segments and triggering the data acquisition for each segment separately. Using this mode the probing LED pulses (Toshiba TLRA 190 P, $\lambda_{max} = 660$ nm) could be distributed nonlinearly in the selected time range. The trigger pulses for DSO, LEDs and microchannel plate detector (MCP-PMT R5916U-51, Hamamatsu) were provided by two pulse generators (Systron-Donner Corporation, Datapulse). These pulse generators were triggered by two function generators (DS345, Stanford Research Systems) operating in series and providing trigger pulses for the fast and slow parts of the signal, respectively. The width of the trigger pulses was determined by the pulse generators to be 4 μs for the MCP-PMT gate and 6 μs for the LED pulses. The gate pulses for the MCP-PMT were delayed by 1 μs with respect to the LED pulses to switch on the detector when the LED emission reached its steady state level.

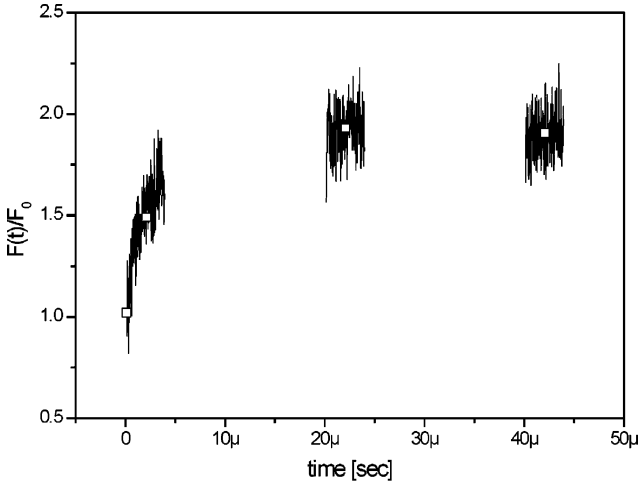

FIGURE 1: First four data points (open squares) and raw signal (noisy curves) of the normalized fast fluorescence rise induced in *A. thaliana* wild type plants by excitation with a saturating laser flash (6.2×10^{15} photons/(cm^2 flash)). The first open square symbol depicts the fluorescence signal at 100 ns. The second to fourth open squares reflect the averaged data points of the respective raw signal (see text).

The sample was excited by actinic flashes ($\lambda = 532$ nm, fwhm = 10 ns) from a frequency-doubled Nd:YAG laser (Spectrum GmbH) with a maximum laser flash energy of 28 mJ/(cm^2 flash) which is equivalent to 7.5×10^{16} photons/(cm^2 flash). To improve the signal-to-noise ratio, 50 single measurements on the same leaf were averaged with a dark time of 90 s between the actinic flashes. It was carefully checked that the kinetics were not altered due to the application of 50 measurements on the same leaf. For a 10 s sweep the raw signal consists of 59 parts of 4 μs duration. The raw data were transferred to a computer via a GPIB-bus (PC IIA, National Instruments) for further data processing.

Data Evaluation. Typical traces of the raw signal are shown in Figure 1 for the first three segments after laser excitation at $t = 0$ s. Each 4 μs signal was averaged in time to yield a single data point symbolized by an open square. As the result of this procedure a graphical representation of the data set is obtained as shown in Figure 2 on a logarithmic time scale. The additional data point at 100 ns (Figure 1) is not the average of a 4 μs signal but is the starting point of the first 4 μs time window after the actinic flash and depicts the time resolution of the system.

THEORETICAL BASIS

In general the normalized time dependent yield of Chl fluorescence $F(t)/F_0$ in plants can be ascribed to two types of emitters: (i) ^{1}Chl* belonging to PS II and its antennae and (ii) ^{1}Chl* that are not coupled to PS II via excitation energy transfer (EET). The general relation is given by

$$\frac{F(t)}{F_0} = \frac{[\Phi_{II}(t) + \Phi_u(t)]I_{abs}}{[\Phi_{II,0} + \Phi_{u,0}]I_{abs}} \quad (1)$$

where Φ are the corresponding fluorescence quantum yields of ^{1}Chl* coupled (index II) or not coupled (index u) via EET

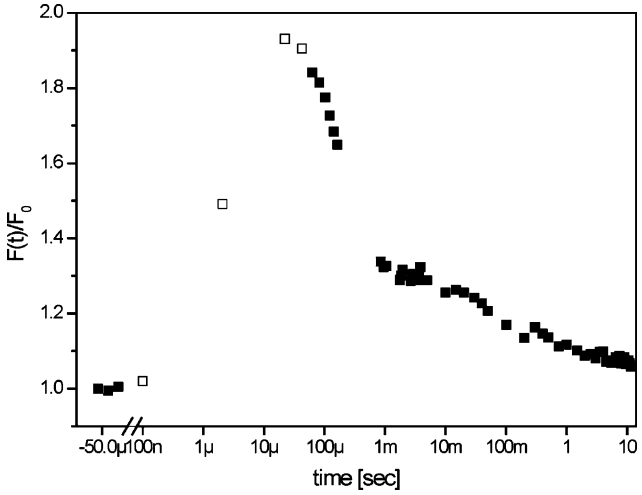

FIGURE 2: Typical traces of flash induced transient change of normalized fluorescence yield induced in *A. thaliana* wild type plants by excitation with a saturating laser flash (6.2×10^{15} photons/(cm^2 flash)). Note the logarithmic time scale. The data points shown in detail in Figure 1 are symbolized by open squares.

to PS II. $F(t)/F_0$ is the experimentally detected normalized fluorescence transient. With $u(t) = \Phi_u(t)/\Phi_{II,0}$ and $u_0 = \Phi_{u,0}/\Phi_{II,0}$, rearrangement of eq 1 leads to the expression

$$\frac{\Phi_{II}(t)}{\Phi_{II,0}} = [1 + u_0]\frac{F(t)}{F_0} - u(t) \qquad (2)$$

where $u(t)$ is a time dependent function that reflects the normalized fluorescence yield of ^{1}Chl* that is not coupled to PS II, i.e., emission from PS I or disconnected LHC. Since the PS I fluorescence yield at room temperature is not only rather small compared to PS II, but also almost invariant to most conditions (*45*), the time course of $u(t)$ can be approximately described by a constant and the kinetics of the flash induced transient population of the quencher ^{3}Car in PS I and disconnected LHC,

$$u(t) = u_0\{1 - [^3\mathrm{Car}]_u e^{-t/\tau_{Car}}\} \qquad (3)$$

where $[^3\mathrm{Car}]_u \leq 1$ is the normalized population of Car triplets that act as quenchers of excited singlet states of Chls that are not coupled to PS II via EET.

Accordingly, two contributions have to be discussed: emission from PS I and pigment protein complexes that are disconnected from EET to PS II. Fluorescence lifetime measurements in the ps/ns time domain revealed that the fraction of disconnected LHC complexes is rather small (Huyer et al., unpublished results). The contribution of PS I to fluorescence emission at room temperature is very small and ignored in almost all studies on light induced fluorescence yield changes. Therefore, for the following analysis we assume that u_0 is sufficiently small, so that the experimentally obtained transient $F(t)/F_0$ practically equals $\Phi_{II}(t)/\Phi_{II,0}$.

The fluorescence signal of the large ensemble of PS II complexes in the thylakoid membrane essentially depends on two parameters: (i) the "connectivity" of the PS II complexes via electronically excited energy transfer and (ii) the time dependent population probability of states that affect the fluorescence quantum yield either as photochemical or nonphotochemical quenchers. In general, the time dependent fluorescence quantum yield of PS II, $\Phi_{II}(t)$, can be described by the following equation:

$$\Phi_{II}(t) = \frac{k_f}{k_f + k_\Sigma + \sum_i k_i[Q_i(t)]} K_{EET} \qquad (4)$$

where k_f is the emissive rate constant of chlorophyll, k_Σ is the sum of all nonradiative deactivation processes, and $[Q_i(t)]$ is the time dependent normalized concentration of quencher i with k_i being the respective rate constant of fluorescence quenching. This equation tacitly implies that the rate constants themselves are time independent. K_{EET} is a structure dependent factor that describes the connectivity of the PS II complexes in terms of electronically excited energy transfer (EET) as described earlier (*46*). For the limiting case of free EET among the PS II complexes, $K_{EET} = 1$.

The extent of EET connectivity gives rise to a nonlinear relationship between quencher concentration and fluorescence emission (*47*). It was shown that fluorescence induction curves of thylakoids from higher plants can be satisfactorily described by an EET probability of about 0.5 (*47*, *48*). Recent studies on tobacco plants led to the conclusion that in terrestrial plants the connectivity between PS II units is high in contrast to some marine species (*49*). Accordingly, the "lake" model of very high PS II connectivity appears to be a suitable model approximation that will be used for the following analysis. For the special case of single turnover flash excitation the transient chlorophyll fluorescence yield is determined by the redox states of the PS II cofactors P680 and Q_A, and the triplet state of Car. The triplet state of chlorophyll is also an efficient quencher. However, the population probability is negligibly small owing to the very rapid transfer to Car (*50*). Likewise, Pheo$^-$ acting as a potent quencher (*51*, *52*) can also be ignored because it becomes reoxidized by Q_A via a 300 ps kinetics (*53*−*55*) that is by far too fast to be detectable in a setup of about 100 ns time resolution.

Starting from the dark adapted state [P680 Q_A Car] with Q_A as the only quencher, the actinic flash generates, within 1 ns, the state [P680$^{+\bullet}$ Q_A^- ^{3}Car], where the quenching state Q_A is replaced by the nonquenching state Q_A^-, and the nonquenching states P680 and Car are replaced by the quenching states P680$^{+\bullet}$ and ^{3}Car. For this "3-quencher" model and $K_{EET} = 1$, eq 4 takes the form

$$\Phi_{II}(t) = \frac{k_f}{k_f + k_\Sigma + k_{Car}[^3\mathrm{Car}(t)] + k_{PC}[Q_A(t)] + k_{P680}[\mathrm{P680}^{+\bullet}(t)]} \qquad (5)$$

Equation 5 reveals that a simulation of the flash induced transients of the chlorophyll fluorescence yield requires information on the time dependence of the quencher concentrations and on the rate constants of all deactivation processes. The time dependence of the normalized quencher concentrations [P680$^{+\bullet}$(t)] and [Q_A(t)] is commonly described

by multiexponential rise or decay kinetics, respectively, of the form

$$[Q_A(t)] = 1 - \sum_i a_{Q,i} \exp(-t/\tau_{Q,i}) - a_{Q,\text{offset}} \quad (6)$$

$$[P680^{+\bullet}(t)] = \sum_j a_{P680,j} \exp(-t/\tau_{P680,j}) \quad (7)$$

where the different exponential components i and j are attributed to the reoxidation of Q_A^- by $Q_B(Q_B^-)$ and the reduction of $P680^{+\bullet}$ by Y_Z, respectively, and $a_{Q,\text{offset}}$ mainly reflects the equilibrium constant of $Q_A^-\ Q_B \leftrightarrow Q_A Q_B^-$ (see ref *56* and references therein).

The normalized concentration of the ^{3}Car state is described by a monoexponential decay kinetics:

$$[^3\text{Car}(t)] = a_{\text{Car}} \exp(-t/\tau_{\text{Car}}) \quad (8)$$

where a_{Car} describes the population of the ^{3}Car state generated by the actinic flash normalized to the $P680^{+\bullet}$ concentration.

The rate constant k_f obtained from the lifetime and quantum yield of chlorophyll in solution was found to be about $(15\ \text{ns})^{-1}$ (*57*). The rate constant k_Σ cannot be determined directly. However, on the basis of the sum of rate constants $k = k_f + k_\Sigma$ that was determined for core complex preparations and solubilized LHC II preparations to be about $(4.3\ \text{ns})^{-1}$ (*58*−*61*), k_Σ is calculated to be about $(6\ \text{ns})^{-1}$. The ratio of the rate constants k_{Car}/k was estimated to be about 7 in solubilized LHC II (*62*). This value can be used as a reasonable estimate for PS II. With respect to the rate constants k_{PC} and k_{P680} it is usually assumed (*63*, *64*) that the quenchers Q_A and $P680^{+\bullet}$ have virtually the same quenching efficiency, i.e., that the ratio k_{P680}/k_{PC} is about 1. As a consequence of this assumption, the formation of $[P680^{+\bullet}\ Q_A^-]$ and its back reaction to $[P680\ Q_A]$ should not be accompanied by a net change of fluorescence yield. Therefore, the latter reaction is assumed to be not detectable by measurements of flash induced fluorescence yield changes. This "dogma" is of central relevance for the interpretation of flash induced fluorescence transients, especially in mutants where the WOC could be disturbed thus giving rise to enhanced contribution of $[P680^{+\bullet}\ Q_A^-]$ recombination to the overall reaction pattern.

Surprisingly, until now the key assumption on a back reaction between $P680^{+\bullet}$ and Q_A^- being not detectable by fluorescence transients lacks any experimental proof. Therefore, this "dogma" has been analyzed as a prerequisite for reliable interpretation of flash induced fluorescence yield changes in *A. thaliana* WT and mutant plants. The results obtained reveal for the first time that the widely accepted idea of a $[P680^{+\bullet}\ Q_A^-]$ back reaction that is not accompanied by transient changes of the fluorescence yield has to be revised.

RESULTS AND DISCUSSION

The $P680^{+\bullet}$ Quenching Efficiency. In order to address the question of determining the ratio of the rate constants k_{P680} and k_{PC}, a suitable sample treatment has to be used where the lifetime of $P680^{+\bullet}$ generated by the actinic flash is sufficiently lengthened to be measurable without interference by other quenchers, especially by ^{3}Car. Since fluorescence yield measurements do not permit a straightforward identification of the nature of a specific fluorescence quencher, an independent method has to be used to verify the inhibition of $P680^{+\bullet}$ reduction by donor side reactions. Measurements of flash induced absorption changes at 830 nm provide a most suitable tool to achieve this goal. In order to avoid interference by contributions due to P700 turnover and to permit a well defined sample treatment that specifically interrupts the $P680^{+\bullet}$ reduction by Y_Z, PS II membrane fragments instead of whole leaves were used for these studies. PS II membrane fragments from spinach were illuminated for 5 min ($255\ \mu\text{mol}/(\text{m}^2\text{s})$ PAR) in the presence of 5 mM hydroxylamine (NH_2OH). This procedure is known to inhibit the electron transfer between $P680^{+\bullet}$ and Y_Z (*65*). In order to avoid destructive effects due to long-term reactions of NH_2OH and in addition to eliminate electron donation of NH_2OH to the donor side of PS II, the sample was diluted 10-fold in buffer solution (MES pH 6.5, no salts) after the CW-PAR illumination, centrifuged, and resuspended in buffer. This important washing step was repeated twice in order to avoid any electron donation by NH_2OH to PS II and $P680^{+\bullet}$ reduction kinetics with lifetimes of tens of microseconds (data not shown).

The upper panel of Figure 3 shows a typical trace obtained for flash induced 830 nm absorption changes in samples prepared by the special NH_2OH treatment. The relaxation of these absorption changes is characterized by a monoexponential $P680^{+\bullet}$ reduction kinetics with a lifetime of about 260 μs. These kinetics are attributed to the back reaction between Q_A^- and $P680^{+\bullet}$ (*66*). The measurement of flash induced absorption change was affected by a low frequency shift of the baseline, and therefore the fit provides only a rough estimation for the lifetime of the observed decay kinetics. When taking into account the baseline shift, a lifetime of 150−200 μs is more realistic for this reaction. Regardless of the baseline problem, the data unambiguously show that no $P680^{+\bullet}$ reduction in the time range of nanoseconds and tens of microseconds takes place in this type of sample preparation.

The corresponding flash induced changes of the fluorescence quantum yield obtained with the same sample preparation are depicted in the lower panel of Figure 3. In order to cover a wide time domain, the transient of the normalized fluorescence yield is presented on a logarithmic time scale.

The flash induced changes of the fluorescence yield are characterized by an unresolved "instantaneous" decrease followed by a rise that could be fitted by a two-exponential kinetics with lifetimes of 4.7 μs and 160 μs and relative amplitudes of 50% for both components. The 4.7 μs kinetics can readily be attributed to the disappearance of the quencher ^{3}Car (*50*, *62*). The lifetime of the slower kinetics exhibits a striking similarity to that of the direct recombination reaction between $P680^{+\bullet}$ and Q_A^- monitored via measurements of 830 nm absorption changes (see Figure 3). It is therefore concluded that the rise kinetics of the slow component (160 μs) of the flash induced fluorescence yield reflect the recombination reaction between $P680^{+\bullet}$ and Q_A^-. As an important consequence of this finding, the widely used dogma of very similar rate constants for photochemical and nonphotochemical fluorescence quenching by Q_A and $P680^{+\bullet}$, respectively, has to be revised, at least for PS II membrane

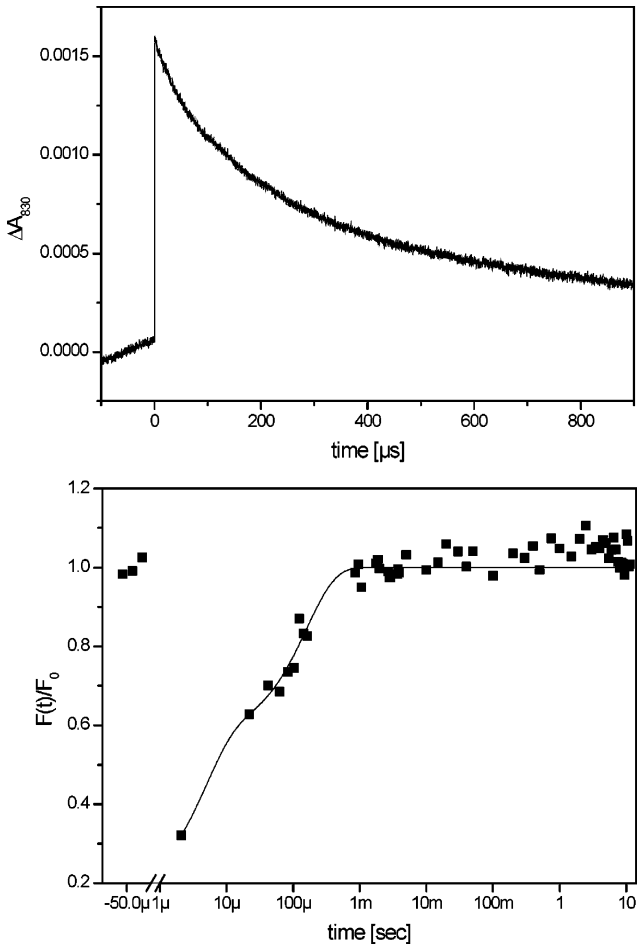

FIGURE 3: Upper panel: Flash induced absorption changes at 830 nm of NH_2OH treated PS II membrane fragments from spinach. Lower panel: Flash induced changes of the normalized fluorescence yield (filled squares) in NH_2OH treated PS II membrane fragments from spinach at maximal laser flash energy (7.5×10^{16} photons/(cm^2 flash)). The time axis is given in a logarithmic scale. The full lined curve represents a fit with a two-exponential model.

fragments after NH_2OH treatment. In general, the idea of a "fluorescence-silent" back reaction between $P680^{+\bullet}$ and Q_A^- has to be abandoned as a basis for the interpretation of flash induced fluorescence transients. Its validity for specific organisms or sample preparations remains to be proven. On the basis of the data of Figure 3, the ratio k_{P680}/k_{PC} has to be considered as a variable that might depend on the sample type and preparation.

An approximate estimation of the ratio k_{P680}/k_{PC} from the data of Figure 3 can be achieved on the basis of eq 5. The value of $\Phi_{II,0}$ is given by the conditions $[^3Car(t = 0)] = [P680^{+\bullet}(t = 0)] = 0$ and $[Q_A(t = 0)] = 1$. Accordingly one obtains

$$\frac{\Phi_{II}(t)}{\Phi_{II,0}} = \frac{k_f + k_\Sigma + k_{PC}}{k_f + k_\Sigma + k_{Car}[^3Car(t)] + k_{PC}[Q_A(t)] + k_{P680}[P680^{+\bullet}(t)]} \quad (9)$$

An inspection of eq 9 readily shows that at a characteristic time t_{P680} where the populations $[^3Car(t)]$ and $[Q_A(t)]$ are sufficiently small and $[P680^{+\bullet}(t)]$ is close to 1, the ratio k_{P680}/k_{PC} can be described by

$$\frac{k_{P680}}{k_{PC}} \approx \frac{\Phi_{II,0}}{\Phi_{II}(t_{P680})} \frac{1 + (k_f + k_\Sigma)/k_{PC}}{1 + (k_f + k_\Sigma)/k_{P680}} \quad (10)$$

These conditions are satisfied with a reasonable approximation at $t \approx 10$ μs: $[^3Car(t)]$ is close to zero owing to the decay kinetics of about 2 μs under aerobic conditions (*50*, *62*); likewise the extent of $[Q_A(t)]$ is also very small since Q_A^- becomes predominantly reoxidized with a rate of a few hundreds of microseconds (*56*); on the other hand, the normalized population $[P680^{+\bullet}(t)]$ formed by a saturating flash is nearly 1 for a 150 μs reduction kinetics in the NH_2OH pretreated sample (see Figure 3).

Furthermore, the photochemical yield of PS II with open reaction center is larger than 0.8, thus implying the relations $(k_f + k_\Sigma)/k_{PC} \ll 1$ and $(k_f + k_\Sigma)/k_{P680} \ll 1$ for $k_{PC} \leq k_{P680}$. When considering the extreme case of $(k_f + k_\Sigma)/k_{PC} \approx 0$, eq 10 simplifies to $k_{P680}/k_{PC} = F_0/F(10\ \mu s)$ and a value of about 1.7 is gathered from the data of Figure 3 for the ratio k_{P680}/k_{PC}. Since this is a lower limit that increases at higher fractions of $(k_f + k_\Sigma)/k_{PC}$, a more realistic number is a value of about 2 for k_{P680}/k_{PC}.

At a first glance it is surprising that the nonphotochemical quencher $P680^{+\bullet}$ is more efficient than Q_A in dissipation of excited singlet states arriving at the $(Chl)_4Pheo_2$ core pigment ensemble of PS II (for a discussion of the nature of P680 see ref *67* and references therein). According to the exciton−radical pair equilibrium model (*21*, *68*, *69*) the exciton trapping in PS II is limited by the stabilization of the primary charge separation via electron transfer from $Pheo^{-\bullet}$ to Q_A. The kinetics of this reaction are characterized by a lifetime of about 300 ps (*53*−*55*). Direct measurements of the lifetime of the excited cation radical $^1(P680^{+\bullet})^*$ are not available, but in general the lifetimes of cation and anion radicals of excited (bacterio)chlorophylls are very short. Furthermore mechanistic studies on the excited state energy transfer from the monomeric bacteriochlorophyll $^1B^*$ to the cation radical P^+ of the special pair in reaction centers of purple bacteria showed this transfer to be as fast as that from $^1B^*$ to P in the ground state and about 5 orders of magnitude faster than expected within the framework of the Förster theory (*70*). An analogous scenario is likely to exist in PS II. Therefore, the faster dissipation of excited chlorophyll singlets by $P680^{+\bullet}$ compared to Q_A is very plausible on the basis of experimental evidence.

It has to be mentioned that a decrease of fluorescence yield in the presence of NH_2OH below the level of F_0 has already been described in a former report (*64*), but was exclusively attributed to the formation of carotenoid triplet states. This conclusion was based on the assumption that the quenching efficiencies for states [P680 Q_A] and $[P680^{+\bullet}\ Q_A^-]$ are virtually the same. As a consequence of our present analysis, the "three quencher model" has to be extended and an additional component introduced into eqs 6 and 7 that accounts for the recombination reaction between $P680^{+\bullet}$ and Q_A^-. The lifetime τ_{recomb} of this component is linked between $[P680^{+\bullet}(t)]$ and $[Q_A(t)]$. On the basis of this extension, the

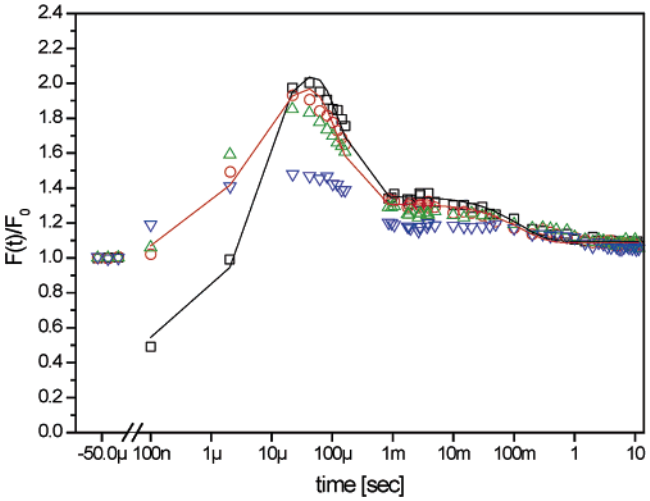

FIGURE 4: Laser flash induced transient changes of the normalized fluorescence yield in whole leaves of wild type plants of *A. thaliana* at different laser flash energies (black squares 7.5×10^{16} photons/(cm^2 flash), red circles 6.2×10^{15} photons/(cm^2 flash), green triangles up 3.0×10^{15} photons/(cm^2 flash), blue triangles down 5.4×10^{14} photons/(cm^2 flash)). Line graphs represent numerical fits at saturating laser flash energies (black line 7.5×10^{16} photons/(cm^2 flash), red line 6.2×10^{15} photons/(cm^2 flash)).

time dependent normalized populations of the quenchers $P680^{+\bullet}$ and Q_A are given by the relations

$$[Q_A(t)] = 1 - (1 - p_{recomb})\sum a_{Q,i} \exp(-t/\tau_{Q,i}) - (1 - p_{recomb})a_{Q,offset} - p_{recomb} \exp(-t/\tau_{recomb}) \quad (11)$$

and

$$[P680^{+\bullet}(t)] = (1 - p_{recomb})\sum a_{P680,j} \exp(-t/\tau_{P680,j}) + p_{recomb} \exp(-t/\tau_{recomb}) \quad (12)$$

where p_{recomb} is the probability that $[P680^{+\bullet} Q_A^{-}]$ recombines via back reaction, $a_{Q,i}$ and $a_{P680,j}$ are normalized amplitudes and $\tau_{Q,i}$ and $\tau_{P680,j}$ are the lifetimes of the corresponding kinetic components, and $a_{Q,offset}$ represents the normalized extent of the nonrelaxing fraction of PS II within the time domain of the measurements (10 s).

It has been shown that in samples with intact oxygen evolution the $P680^{+\bullet}$ reduction can be satisfactorily described by three-exponential kinetics in the ns/μs time domain. This general feature was observed in thylakoids (*71*), PS II membrane fragments (*72*−*74*) and PS II core complexes (*75*) from spinach, and PS II core complexes from the thermophilic cyanobacterium *Thermosynechococcus elongatus* (*75*). It is therefore most likely that the $P680^{+\bullet}$ reduction in *A. thaliana* leaves can be fitted by an analogous three-exponential kinetics. On the basis of this idea the fits of the fluorescence data were performed with the lifetime of the three components fixed at 30 ns, 300 ns, and 35 μs. This approach was shown to be justified by different fits of the experimental data of Figure 4 which reveal that the quality of the description does not critically depend on the exact time constants (data not shown). The kinetics of about 300 ns become blurred by the overlapping signal originating from the turnover of ^{3}Car. As a consequence, a satisfying fit can be also achieved if the time course of $P680^{+\bullet}$ reduction is approximated by a two-exponential fit. However, in this case the amplitude values of the ^{3}Car are less reliable numbers (vide infra).

The relationship for $[Q_A(t)]$ under conditions of negligibly small values of p_{recomb} has been described by a three-exponential decay kinetics ($i = 3$ with f = fast, m = middle, and s = slow) for thylakoids and PS II membrane fragments from spinach (*56*).

This extended "3-quencher" model formally separates the probability of the recombination reaction, p_{recomb}, from the overall reaction of $P680^{+\bullet}$ reduction by Y_Z and Q_A^- reoxidation by $Q_B(Q_B^-)$. It should be mentioned that under native conditions the amplitude of the recombination reaction is expected to be rather small (vide infra), i.e., the contribution of this component can usually be neglected in a first approximation. The ratio of the rate constants k_{P680}/k_{PC}, however, directly affects the quantitative data interpretation. Whereas in the case of $k_{P680}/k_{PC} = 1$ the amplitudes of fluorescence yield changes linearly reflect the quencher concentrations, this is not true when k_{P680}/k_{PC} attains values of about 2.

Fluorescence Yield Changes in Whole Leaves of A. thaliana. The transients of flash induced fluorescence yield changes of whole leaves of *A. thaliana* wild type plants were measured at four different energies of the actinic flash (the energy is a direct measure of the photon density per flash and unit area, i.e., $N = 7.5 \times 10^{16}$ photons/(cm^2 flash) for 28 mJ/(cm^2 flash) at 532 nm). An inspection of the results obtained and summarized in Figure 4 reveals that the actinic flash induces an "instantaneous" change within 100 ns. The extent and sign of this change, $F_{100\,ns}/F_0$, strongly depend on the energy of the actinic flash. This first event is followed in all traces by a fast fluorescence rise to a maximum of the normalized fluorescence yield F_M/F_0. Its value also increases with increasing laser flash energy. A maximum value of about 2 is reached within 50 μs at the highest energy of the actinic flash. At longer times the normalized fluorescence yield declines in the time range of hundreds of microseconds and in the millisecond time range. Even 10 s after the actinic laser flash the fluorescence yield is not relaxed to its original value F_0. The most pronounced dependency on the energy of the actinic flash is observed for $F_{100\,ns}/F_0$ and the subsequent fast fluorescence rise in the time range from 100 ns to about 50 μs (F_M/F_0). At lower laser flash energies the fluorescence yield change at $t = 100$ ns ($F_{100\,ns}/F_0$) exhibits an unresolved increase that decreases in its extent with increasing laser flash energy, even falling below unity at the highest laser flash energy (see also Figure 5).

This characteristic dependence of the "instantaneous" change of the fluorescence yield is known to originate from the quenching by ^{3}Car states and has been described in a previous study (*39*) on thylakoid preparations from spinach. Quantitatively, however, the extent of this quenching is somewhat larger than in spinach thylakoids, and as a consequence the normalized fluorescence at $t = 2$ μs, $F_{2\,\mu s}/F_0$, is strongly quenched. This observation suggests that a higher amount of ^{3}Car is formed in whole leaves of *A. thaliana* provided that the quenching efficiency of ^{3}Car is practically the same in both sample types. The maximal fluorescence yield F_M/F_0 exhibits the expected behavior, reaching a saturation value of $F_M/F_0 \approx 2$ at an actinic flash energy of 6.2×10^{15} photons/(cm^2 flash).

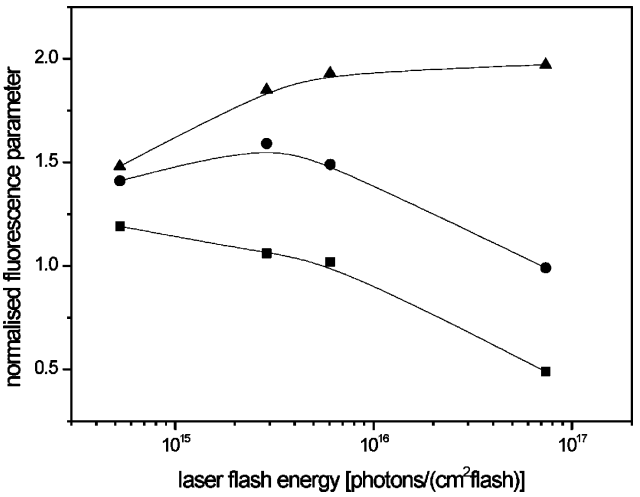

FIGURE 5: Normalized fluorescence parameters $F_{100\,ns}/F_0$ (squares), $F_{2\,\mu s}/F_0$ (circles), and F_M/F_0 (triangles) as a function of laser flash energy.

The numerical data evaluation requires further information on the rate constants k_{P680} and k_{Car}. However, no reliable experimental data are available for these rate constants. Accordingly reasonable assumptions have to be used. In the most simple case k_{P680} and k_{Car} are taken as to be the same. This idea, which appears to be rationalized by the fact that ^{3}Car and $P680^{+\bullet}$ both act as nonphotochemical quenchers with comparable relaxation pathways, is supported by the finding of a value of about 2 for the ratio k_{P680}/k_{PC} that implies the relation $k_{P680} \approx k_{Car}$ (vide supra).

Since the model described above is overparametrized, additional constraints have to be introduced to fit the data. These are based on data reported in the literature for the kinetics of $P680^{+\bullet}$ reduction and Q_A^- reoxidation as outlined above. With respect to the amplitudes of $P680^{+\bullet}$ reduction and Q_A^- reoxidation, it is straightforward to normalize the amplitudes so that $a_{P680,f} + a_{P680,m} + a_{P680,s} = 1$ and $a_{Q,f} + a_{Q,m} + a_{Q,s} + a_{Q,offset} = 1$. It should be kept in mind that the *a* values are relative amplitudes that directly account for the quencher concentration and are not the amplitudes of the fluorescence change as used in previous studies. The lifetime τ_{Car} of the ^{3}Car states was fixed to 2 μs. This value corresponds with recent findings reported for air saturated solubilized LHC II trimers (*50*). In addition to these simplifications of the fit procedure, also the lifetime τ_{recomb} for the recombination reaction was fixed to 160 μs (vide supra). The data were fitted by using eq 9 for the normalized fluorescence yield. Numerical fits were only performed for fluorescence transients that were measured with actinic flashes of energies that saturate the photosynthetic electron transport (i.e., 7.5×10^{16} photons/(cm^2 flash) and 6.2×10^{15} photons/(cm^2 flash)).

The fit of the experimental curves revealed that a singular set of parameters could not be obtained owing to the multitude of parameters in eqs 11 and 12. Several fits with different assumptions were performed. It turned out that replacement of the three-exponential decay kinetics of both $[P680^{+\bullet}(t)]$ and $[Q_A^-(t)]$ by two-exponential kinetics did not affect the quality of data description (see Table 1). Likewise the probability of the recombination reaction between $P680^{+\bullet}$ and Q_A^- could be varied between 0 and 0.1 without serious affects on the fit quality (data not shown). Independent information on the value of p_{recomb} is obtained from the probability of misses for the redox transitions in the WOC. If one assumes that this probability of misses gathered from data fit of the period four oscillation of the flash induced oxygen yield within the framework of the conventional Kok model (*76*) predominantly originates from $[P680^{+\bullet}\ Q_A^-]$ recombination, p_{recomb} values of the order of 0.1 (0.06−0.08) are most likely for thylakoids from higher plants (*12*) and thermophilic cyanobacteria (*77*). In the following fit procedures a value of 0.07 is used.

Table 1 compiles the results gathered from the numerical data analysis with fixed parameters of $p_{recomb} = 0.07$, $k_{P680}/k_{PC} = 2$, $k_{P680}/k_{Car} = 1$ and different assumptions on the kinetics of $P680^{+\bullet}$ reduction and Q_A^- reoxidation. The data sets show that a fully consistent description is achieved for the properties of the PS II reaction pattern in intact leaves of wild type *A. thaliana* plants. Except for a_{Car}, the amplitudes and lifetimes of the free running parameters are invariant to a change by more than 1 order of magnitude of the energy of the actinic flash. This is the expected feature for the reaction behavior of PS II excited by a short (single turnover) flash with an energy above the saturation level of photosynthesis. Furthermore, the values of about 0.8 for the normalized amplitudes of $P680^{+\bullet}$ reduction via nanosecond kinetics nicely fit with data gathered from 830 nm absorption changes induced by a single laser flash in dark adapted PS II membrane fragments from spinach (*72*, *73*). The a_{Car} values show that—apart from the radiative (fluorescence) and nonradiative pathways that are not monitored by our measuring device (see Materials and Methods)—the excess population of excited Chl singlets decays to a significant extent via Car triplets transiently formed as a result of fast energy transfer from ^{3}Chls (owing to intersystem crossing of ^{1}Chl*)

Table 1: Parameters for Transients at 7.5×10^{16} photons/(cm^2 flash) and 6.2×10^{15} photons/(cm^2 flash) Estimated from Numerical Fits Based on Different Model Assumptions (See Text)[a]

photon density [10^{16} photons/(cm^2 flash)]	$a_{P680,f}$	$a_{P680,m}$	$a_{P680,s}$	$a_{Q,f}$	$\tau_{Q,f}$ [μs]	$a_{Q,m}$	$\tau_{Q,m}$ [ms]	$a_{Q,s}$	$\tau_{Q,s}$ [ms]	$a_{Q,offset}$	a_{Car}
7.5	0.67	0.15	0.18	0.59	160	0.09	2*	0.21	600*	0.11	0.75
0.6	0.63	0.18	0.19	0.59	120	0.13	2*	0.18	600*	0.10	0.11
7.5	0.82		0.18	0.62	160	0.05	10*	0.21	200*	0.12	0.84
0.6	0.82		0.18	0.66	130	0.06	10*	0.17	200*	0.11	0.23
7.5	0.82		0.18	0.57	140	0.12	2*	0.20	600*	0.12	0.83
0.6	0.83		0.17	0.59	120	0.13	2*	0.18	600*	0.10	0.24
7.5	0.81		0.19	0.64	160			0.24	133	0.12	0.83
0.6	0.82		0.18	0.68	140			0.22	144	0.11	0.23

[a] Amplitudes are given in relative units. An asterisk (*) indicates parameters that have been fixed.

followed by rapid radiationless decay of ^{3}Car with a lifetime of about 2 μs under aerobic conditions (*50*). The numbers of a_{Car} in Table 1 indicate that the extent of transient ^{3}Car population increases by factors of about 4−7 when the photon flux density of the actinic flash increases by a factor of about 12 above the saturation level of photosynthesis.

A closer inspection of the data however reveals that—regardless of the assumptions used for the (middle) slow component(s) of Q_A^- reoxidation—virtually the same values are obtained for $a_{P680,f}$, $a_{Q,f}$, and $\tau_{Q,f}$. It is important to note that inclusion of a 300 ns component ($\tau_{P680,m}$) does not affect the amplitudes $a_{Q,i}$ or $a_{P680,s}$, but only separates the value obtained for $a_{P680,f}$ from the approximation of $P680^{+\bullet}$ reduction by a two-exponential kinetics into amplitudes $a_{P680,f}$ and $a_{P680,m}$ for the fast and middle components, respectively. However, considering the ratio of the extent of ^{3}Car formation at the two laser flash energies, a_{Car} seems to be more realistic when the 300 ns component is taken into account. Accordingly, since the fit with a three-exponential kinetics of $P680^{+\bullet}$ provides the more appropriate and realistic approach for numerical data evaluation, it will be used for interpretation of the experimental results obtained with lipid mutants (*1*). The parameters compiled in Table 1 indicate the following: (i) after excitation with a single turnover flash the reduction of $P680^{+\bullet}$ by Y_Z in dark adapted leaves of the wild type plants occurs predominantly (about 80%) via nanosecond kinetics ($a_{P680,f} + a_{P680,m}$), (ii) the reoxidation of Q_A^- is dominated by a reaction with a lifetime of the order of 150 μs that is ascribed to the electron transfer in PS II complexes that contain a Q_B site occupied by PQ, and (iii) the fraction of Q_A^- still remaining reduced 10 s after the actinic flash is about 10% of the initially formed Q_A^-.

The values obtained for the total extent of nanosecond kinetics of $P680^{+\bullet}$ reduction are in correspondence with the results gathered from measurements of 830 nm absorption changes of isolated PS II preparations from thermophilic cyanobacteria and higher plants (*72*−*75*). Likewise the values of $a_{Q,f}$ and $\tau_{Q,f}$ are in agreement with measurements of isolated thylakoids and PS II membrane fragments (see ref *56* and references therein). On the basis of this good correspondence it can be concluded that the isolation procedures do not markedly affect the reaction pattern of PS II compared with that of intact leaves and, vice versa, that the fluorometric method described in this study provides a very useful analytical tool for analyzing PS II in intact plant material. This kind of "calibration" test is the basis for studying the properties of mutants where no preparations are available for using more sophisticated spectroscopic methods. The results obtained for *A. thaliana* plants with modified lipid content and composition will be described in a second publication (*1*).

CONCLUDING REMARKS

This study describes new equipment for monitoring laser flash induced transients of the fluorescence quantum yield in the wide range from 100 ns to 10 s that can be successfully used for analyzing the PS II reaction pattern in whole leaves of *A. thaliana* plants. It is also shown for the first time that the currently used dogma of virtually the same quenching efficiency of $P680^{+\bullet}$ and Q_A^- has to be revised. As a consequence, the "3-quencher" model (^{3}Car, $P680^{+\bullet}$, Q_A) is not sufficient for a consistent description of the data and needs to be extended by an explicit inclusion of the probability of back reaction between $P680^{+\bullet}$ and Q_A^-. This is of special relevance for the analysis of mutants with impaired linear PS II electron transport.

SUPPORTING INFORMATION AVAILABLE

Instrument and circuit diagram. This material is available free of charge via the Internet at http://pubs.acs.org.

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