The Polyphasic Rise of Chlorophyll Fluorescence upon Onset of Strong Continuous Illumination:
II. Partial Control by the Photosystem II Donor Side and Possible Ways of Interpretation

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Z. Naturforsch. 42c, 1255—1264 (1987); received June 22, 1987

Chlorophyll Fluorescence, Photosystem II, Photosynthesis, Photochemical Quenching

The fluorescence rise kinetics in saturating light display two well separated components with largely different properties. The rapid rise from $F_0$ to a first intermediate level, $I_1$, is photochemically controlled, while the following phases leading to a secondary intermediate level, $I_2$, and to a peak level, $P$, are limited by thermal reactions. Treatments which primarily affect components at the photosystem II donor side are shown to increase quenching at $I_1$ and/or to suppress the secondary fluorescence rise to $I_2$. Preillumination by single turnover saturating flashes causes $I_1$-quenching oscillating with period-4 in dependence of flash number. It is suggested that this quenching correlates with $(S_3 + S_3)$ states of the watersplitting enzyme system. Suppression of the secondary, $I_1$-$I_2$ rise component is invariably found with treatments which lower electron donation rate by the watersplitting system and are known to favor the low potential form of cyt b 559.

Three different mechanisms are discussed on the basis of which donor-side dependent quenching could be interpreted: 1) Non-photochemical quenching by accumulation of the $P$ 680° radical cation. 2) Dissipative photochemical quenching at a special population of PS II centers (β- or non-B centers) displaying low donor capacity and high rates of charge recombination. 3) Dissipative photochemical quenching via cyclic electron flow around PS II, involving alternate donors to $P$ 680° (like cyt b 559 or carotenoid in their low potential forms), which can compete when donation rate from the water splitting system is slowed down. The possibility of donor-side limitation also being involved in “energy dependent” quenching is discussed.

Introduction

Chlorophyll fluorescence in vivo is controlled by photochemical and non-photochemical quenching mechanisms [1]. In order to obtain reliable information from chlorophyll fluorescence under in vivo conditions, it is essential to differentiate between the different quenching components. This can be achieved by the light-doubling or saturation pulse method [2—4]. The rationale of this method is that under any given condition, application of sufficiently intense light will cause a transient, complete reduction of PS II acceptors and a correspondent complete suppression of photochemical quenching.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyleuca; PS, photosystem; Tris, tris (hydroxymethyl) aminomethane; ANT-2p, 2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene; CCCP, carbonylcyanide-m-chlorophenyl-hydrazone; $Q_A$, primary stable photosystem II acceptor; $Q_B$, secondary PS II acceptor.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen 0341—0382/87/1100—1255 S 01.30/0

As was shown in the preceding paper [5], the fluorescence increase induced by very intense light displays complex kinetics. Three basically different rise components were distinguished:

1) A rapid phase, $F_{0} - I_1$, controlled by the rate of charge separation at PS II centers, corresponding to Delosme’s “photochemical phase” [6].
2) A slower rise with two subphases to a second intermediary level, $I_2$, which is separated from the rapid phase by a dip, $D$, and the rate of which saturates at about 500 W/m².
3) A third phase, amounting to about 15% of total variable fluorescence, which was interpreted to reflect the removal of static fluorescence quenching upon reduction of the PO-pool [7].

With the observed separation between photochemical and thermal rise components [5, 6] a number of questions were raised which relate to the rationale of the saturation pulse method: Is the quenching at $I_1$ photochemical or non-photochemical? Is the primary acceptor, $Q_A$, completely reduced at $I_1$ or at $I_2$? Can one be assured that under all
In the preceding contribution two seemingly contradictory observations were reported [5]. On the one hand, the saturation behavior of \( I_1 \) with increasing light intensity and decreasing temperature pointed to maximal \( Q_A \)-reduction at \( I_1 \). On the other hand, DCMU-type inhibitors raised \( I_1 \) to the \( I_2 \)-level, suggesting a rapid, DCMU-sensitive pathway for \( Q_A \)-reoxidation.

Here, we wish to report on measurements of the fluorescence rise kinetics in saturating light under conditions, which are known to primarily affect the properties of the PS II donor side. It will be shown that the donor side exerts strong control on quenching at \( I_1 \) and on the fluorescence rise to \( I_2 \).

**Materials and Methods**

The experiments were carried out with intact leaves of greenhouse-grown spinach and isolated spinach chloroplasts, either with intact envelopes or osmotically shocked, as previously described [5].

The apparatus for measuring chlorophyll fluorescence induction kinetics in saturating light was based on a pulse modulation system (PAM Chlorophyll Fluorometer, H. Walz, Effeltrich, FRG) [4, 8]. Strong actinic light was applied via an electromagnetic shutter, as described before [5]. Single turnover saturating flashes were obtained from a xenon flash lamp (XST 103, Walz) triggered at 1 Hz by the PAM 103 control unit (Walz).

For other experimental conditions, see legends of the figures.

**Results and Interpretation**

The donor side properties of PS II can be influenced by a variety of treatments and chemical additions (for reviews, see ref. [9–11]), including preillumination to establish certain S-states [12, 13], Tris-treatment [14] and heat-treatment [15], ADRY-reagents [16] and artificial electron donors substituting for the watersplitting enzyme system [17]. In view of the results of the preceding paper [5] it was of interest to investigate the effects of these donor side treatments on the fluorescence rise kinetics in saturating light.

**Preillumination by saturating flashes**

Fig. 1 shows the effect of preillumination by single turnover saturating flashes on the fluorescence rise kinetics in saturating light. The induction pattern is dependent on the number of preilluminating flashes: Preillumination causes suppression of the \( I_1 \)-level, paralleled by elimination of the dip phase (\( I_1 \)-D) and by stimulation of the secondary rise component (D-I\(_2\)), most pronounced after 2 and 6 flashes. When \( O \)-\( I_1 \) is decreased in amplitude, there is a complementary increase in the amplitude of D-I\(_2\), which is primarily occurring within the first sub-phase (see ref. [5]). A period-4 oscillation is apparent from a plot of \( I_1 \) versus the number of preilluminating flashes (Fig. 2). At the same time the \( I_2 \)- and \( P \)-levels are not affected by the flashes. A less pronounced variation in the \( F_0 \)-level appears to be out of phase with the \( I_1 \)-oscillation. Plots of the “photochemical phase” (\( F_0 \)-\( I_1 \)) and of the “thermal phase” (D-I\(_2\)) display antiparallel period-4 oscillations (Fig. 3).

Fig. 1. Fluorescence rise kinetics in saturating light with varying number of preilluminating flashes. Intact spinach chloroplasts, 20 °C. Samples were dark-adapted before preillumination by the indicated number of single turnover saturating flashes at 1 Hz. About 1 s after the last flash of a sequence, measuring light (10 mW/m²) was switched on and within 1 s thereafter actinic illumination (3500 W/m²) was initiated. The characteristic fluorescence levels are indicated (\( F_0 \), \( I_1 \), D, \( I_2 \)) in the control curve (without preillumination). Note: The P-level is not recorded within the given time of about 40 ms (see ref. [5]). One relative unit of fluorescence yield corresponds to the yield at the \( F_0 \)-level.
Fig. 2. Characteristic fluorescence levels of an induction curve in saturating light in dependence of the number of preilluminating flashes. For conditions, see Fig. 1.

Fig. 3. Amplitude of the photochemical phase \( F_0-I_1 \) and of the main thermal phase \( D-I_2 \) on the number of preilluminating flashes. For conditions, see Fig. 1.

These data suggest that the state of the water-splitting enzyme system can control quenching at the \( I_1 \)-level and the consequent fluorescence change during the \( I_1-D-I_2 \) transient. Most quenching at \( I_1 \) is observed, when a high population of \( (S_2 + S_3) \) is established before application of the saturating continuous light. This \( S \)-state dependent quenching may be discussed in connection with former results of Joliot and Joliot [18] and of Delosme [19]:

Joliot and Joliot [18] measured the light induced fluorescence rise at \(-50^\circ\text{C}\), following a variable number of preilluminating saturating flashes at room temperature and fixing of the \( S \)-states by rapid cooling. They found a biphasic rise, with the amplitude of the rapid component being minimal after 2 and 6 preilluminating flashes. Delosme [19] applied a rapid measuring technique to determine the fluorescence yield reached during the last of a series of \( \mu \text{sec} \) flashes. He observed a period-4 oscillation with minima following 2 and 6 preilluminating flashes.

A common feature of these results [18, 19] is that maximal quenching is observed when \( (S_2 + S_3) \) is established by preillumination and that the monitoring of this quenching involves one additional charge separation without the possibility for stabilization, \textit{i.e.} for \( S \)-state advancement \( (S_2 + S_3 \rightarrow S_2' + S_3' \rightarrow S_4 + S_5) \). Hence, it may be concluded on the basis of the identical period-4 pattern, that flash-dependent fluorescence quenching at \( I_1 \) is also representative of \( (S_2' + S_3') \), and that the fluorescence rise to \( I_1 \) involves a single charge separation per center.

**Treatments disconnecting the watersplitting system**

Tris-washing can be used for a relatively mild and selective disconnection of the water splitting enzyme system from the PS II reaction center [14]. Fig. 4 shows the effect of increasing periods of Tris-washing on the fluorescence rise kinetics in saturating light. Tris-washing causes suppression of variable fluorescence, primarily affecting the slower rise component. In Fig. 5 the change of the characteristic fluorescence levels with increasing washing times is plotted. Two different effects can be distinguished. Up to 30 min washing time, the \( I_1 \)-level is not affected, while the \( I_2' \) and \( P \)-levels are decreased. At the same time there is some increase in \( F_0 \). With washing times exceeding 30 min also \( I_1 \) is lowered and eventually the \( D-I_2-P \) rise is substituted by a fluorescence decrease.

A similar behaviour is found with heat-pretreatment. Fig. 6 shows induction curves recorded following exposure of intact chloroplasts to different temperatures for 5 min. All measurements were at \( 20^\circ\text{C} \) after 5 min recovery. As with Tris-washing, also heat-pretreatment causes strong suppression of variable fluorescence. However, there is less selectivity.
with respect to the elimination of the D-I₂ phase: Following heat-pretreatment suppression of D-I₂ is paralleled by a decrease in I₁ and an increase in F₀ (see plot of characteristic fluorescence levels in Fig. 7).

Tris-washing as well as heat-treatment are known to cause a release of Mn from the water splitting enzyme complex [20, 21]. As a consequence, electron donation to PS II reaction centers is slowed down. Also transformation of cyt b 559 HP to LP form has been reported [22]. These two aspects may be important for the interpretation of fluorescence quenching at the I₁-level (see Discussion).

Hydroxylamine also disconnects the water splitting system from the PS II reaction center [23] and causes LP-formation of cyt b 559 [24]. However, hydroxylamine also acts as an artificial donor for PS II [25]. As shown in Fig. 8 hydroxylamine affects the induction kinetics in a distinctly different way than Tris- or heat-treatment. The main effect is a quenching at I₁; the O-I₁ phase is suppressed to almost half

**Fig. 4.** Effect of Tris-washing on the fluorescence rise in saturating light. Intact spinach chloroplasts were broken in 50 mM Tris, pH 8 and diluted with a buffer containing 800 mM Tris-KOH at pH 8 to a chlorophyll concentration of about 0.25 mM. Following the incubation times indicated in the figure, aliquots were suspended in the cuvette in the usual reaction buffer and 1 min thereafter the induction curves were recorded.

**Fig. 5.** Characteristic fluorescence level of an induction curve in saturating light in dependence of the duration of a Tris-washing pretreatment. Conditions as described in Fig. 4.

**Fig. 6.** Effect of the pretreatment temperature on the fluorescence rise kinetics in saturating light. Aliquots of an intact spinach chloroplast stock suspension were pretreated for 5 min at the indicated temperatures and then rapidly diluted in suspension buffer at 20 °C. 5 min thereafter the rise kinetics were recorded.
of its control amplitude. On the other hand, there is no suppression of the I₁-D-I₂-P part of the induction curve, except that the first subphase of D-I₂ is slowed down.

**ADRY reagents**

ADRY reagents accelerate the deactivation of S₂ and S₃ [16] and induce transformation of cyt b 559 HP to LP form [26]. In Fig. 9 and 10 the effects of the ADRY reagents ANT-2p and CCCP on the fluorescence rise kinetics in saturating light are shown. With increasing ANT-2p concentration, there is first a suppression of the D-I₂ rise phase, paralleled by enhancement of the I₁-D dip phase (Fig. 9). At concentrations exceeding $5 \times 10^{-6}$ m (with the given chlorophyll concentration), also the I₁-level is suppressed, along with some lowering of the Fₐ-level, which suggests static fluorescence quenching by ANT-2p. With CCCP, as with ANT-2p, the D-I₂ phase is eliminated, while the I₁-D dip is stimulated up to $10^{-3}$ m CCCP (Fig. 10). However, contrary to ANT-2p, CCCP causes a substantial increase of the I₁-level, accompanied by some rise in Fₐ.

Common to both ADRY reagents is the suppression of the D-I₂ rise and stimulation of the I₁-D dip. The CCCP effect on I₁ is similar to that observed with DCMU-type inhibitors [5]. Indeed, binding of CCCP to the B-protein has been reported [27].

These results confirm that the D-I₂ phase is related to the activity of the water splitting enzyme system. With the extremely high light intensity used in this type of experiment, high concentrations of the ADRY reagents are required, so that the rate of deactivation can compete with the rate of S-state advancement [16].

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**Fig. 7.** Characteristic fluorescence levels of an induction curve in saturating light in dependence of the temperature of a 5 min pretreatment. For conditions, see Fig. 6.

**Fig. 8.** Effect of NH₂OH on the fluorescence rise kinetics in saturating light. Curves were recorded 5 min following chloroplast suspension in presence or absence of NH₂OH. Intact spinach chloroplasts, 20 °C.

**Fig. 9.** Effect of ANT-2p on the fluorescence rise kinetics in saturating light. Broken chloroplasts, class D, at a chlorophyll concentration of 100 µg/ml. Samples were incubated with ANT-2p at the indicated final concentrations for 2 min before curve recordings.
**Extreme pH-values**

Reactions at the PS II donor side involve protolytic steps and pH-dependent rate constants [9–11]. Water splitting activity is known to become inhibited at pH-values above 8.5 and below 5 [28, 29].

In Fig. 11 the effect of pH on the fluorescence rise kinetics in saturating light is displayed. Fig. 12 shows the dependence of the half-rise time of the D-I₂ phase on pH. It is apparent that the rate of this “thermal” phase is constant at pH values between 5 and 8. Below pH 5 and above pH 8 the rate is decreased. As

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**Fig. 10.** Effect of CCCP on the fluorescence rise kinetics in saturating light. Broken chloroplasts, class D, at a chlorophyll concentration of 100 μg/ml. Samples were incubated with CCCP at the indicated final concentrations for 2 min before curve recordings.

**Fig. 11.** Effect of pH on the fluorescence rise kinetics in saturating light. Intact chloroplasts were osmotically shocked in 5 mM MgCl₂ and resuspended isotonically in 330 mM sorbitol, 50 mM MES-HEPES-Tricine KOH buffers adjusted to the indicated pH values, 10⁻³ m Gramicidin was added to assure pH equilibration between the medium and the internal thylakoidal space. Curves were recorded 1 min following re-suspension.

**Fig. 12.** pH-dependency of the half-rise time of the D-I₂ phase in the fluorescence rise upon illumination with saturating light. Conditions as in Fig. 11.
with ADRY reagents, Tris- and heat-treatment, stimulation of the I, D dip phase, accompanied by suppression of D-I, is observed at high pH values.

Discussion and Conclusions

The presented results clearly demonstrate a major control of the fluorescence induction kinetics in saturating light by properties of the PS II donor side. This donor side control has to be reconciled with the observed effect of DCMU-type inhibitors [5] known to act at the PS II acceptor side.

The fluorescence quenching at the I, level was shown to be modulated by the S-state distribution, and it was suggested that this quenching corresponds to the S-state dependent quenching already explored by Joliot and Joliot [18] and Delosme [19]. However, the effects of Tris- and heat-treatment, as well as of ADRY reagents and NH$_2$OH, indicate that quenching at I, is also favored by conditions which prevent formation of (S$_2$ + S$_3$). Hence, a quenching mechanism must be involved, which is enhanced by (S$_2$ + S$_3$) without depending on it.

The “thermal phase”, D-I, of the fluorescence rise was shown to be suppressed by treatments which affect the water splitting enzyme system. In presence of an alternate source of electrons, like NH$_2$OH, the D-I, phase is maintained. Therefore, it may be concluded that the removal of quenching in the course of the D-I, phase is dependent on water splitting activity, unless an artificial donor system is available. A special case is given with the effect of ANT-2p, which is supposed to catalyze rapid electron donation to P 680$^+$ [31]. Possibly, the substance by which oxidized ANT-2p is re-reduced is identical to that which also re-reduces P 680$^+$ when water splitting activity is low.

The results presented in the preceding contribution [5] indicated a partial control of the quenching at I, and of its removal during the D-I, phase by the PS II acceptor side. The present contribution strongly supports a control by the PS II donor side. These apparently conflicting findings have to be reconciled. In the following section possible ways of interpretation will be discussed:

$P$ 680$^+$-quenching

It is known that P 680$^+$ can trap excitation energy and, hence, can act as a fluorescence quencher [30]. If P 680$^+$ would partially accumulate upon illumination with continuous, saturating light, this could explain quenching at I, despite full reduction of Q$_A$. Indeed, all treatments shown above to increase quenching at I, and to suppress the D-I, rise, are also known to slow down the reduction rate of P 680$^+$ by the donor side, except for the effect of ADRY reagents (but see above considerations on the catalytic function of ANT-2p). It could be argued that DCMU prevents quenching at I, by not allowing more than one turnover per center. Possibly, the donor side limitation will only show when rapid successive charge separation at the same center occurs and the primary donors to P 680$^+$ become partially exhausted. Following a single turnover flash, P 680$^+$ is re-reduced rapidly with half-life times of 20, 50 and 260 ns [32]. However, with repetitive flash-illumination a 35 μsec phase is observed [33], which is maximal in the states (S$_2$ + S$_3$) [34]. In continuous light, after the first secondary donors have become exhausted, donation from the water splitting system in its different S-states becomes limiting, and, hence, S-state dependent donation rates and damage to the enzyme system should become more clearly expressed in P 680$^+$ formation and corresponding fluorescence quenching. Hence, in principle the development of P 680$^+$ quenching under the conditions of illumination by saturation pulses appears possible.

There are, however, also aspects which argue against a direct involvement of P 680$^+$ quenching at I,. The observed oscillation pattern of I, in dependence of the number of preilluminating flashes is identical to that described by Delosme [19] and Joliot and Joliot [18], in which cases there can be no doubt that the actual fluorescence measurement does not induce more than a single turnover at PS II centers. Hence, we should conclude that at I, also not more than one turnover per center has taken place, and, if this is so, rapid donation by the first secondary donors should prevent P 680$^+$ formation. Furthermore, it appears that the same type of quenching, which controls I,, may also limit the fluorescence increase which can be brought about by a single saturating flash [5, 19].

PS II heterogeneity

Different types of PS II heterogeneity have been described in past work (for reviews, see ref. [35–36]). Any of the reported heterogeneities could be reflected in the polyphasic rise of fluorescence in
saturating light. A complete discussion of this aspect is out of the scope of this paper. We would like to concentrate here on the possibility that quenching at I₁ is caused by a special population of PS II centers which displays an unusually low donation rate to P 680⁺. These could be β- or non-B-type reaction centers with Q₂ [37], Xₐ [38] or X [39] as acceptors, a common property of which appears to be a half-time of 35 μs for recombination [35]. As 35 μs luminescence oscillates with a periodicity of four in a flash series [40], it also appears possible that the relative amount of this type of centers is dependent on the S-states. Van Gorkom [35] considers the possibility that centers may be able to switch from Qₐ reduction to X reduction, e.g. under the influence of a low internal pH. With respect to the interpretation of our results, it could be argued that quenching at I₁ is caused by rapid recombination of P 680⁺ X⁻, i.e. by a kind of dissipative photochemical shortcircuit, or by P 680⁺ quenching, or by both. In both cases, to explain the DCMU effect (suppression of quenching at I₁) one would have to assume that DCMU interferes either with the formation of P 680⁺ X⁻ or with the recombination reaction.

Alternate donor to P 680⁺

When normal electron donation from the water splitting system is slow or inhibited, P 680⁺ can be also reduced by alternate donors. It is known that cyt b 559 [41, 42] as well as a carotenoid [42, 43] can act in this way. While measurements at cryogenic temperatures revealed large yields of oxidized cyt b 559 [41] or carotenoid radical cation [43], measurements at room temperature show only small changes, unless ADRY reagents or lipophylic anions are present [42, 43]. Both, for cyt b 559 and for the carotenoid it has been argued that formation of low potential forms is essential for effective electron donation to P 680⁺. It appears possible that the oxidized alternate donors are rapidly re-reduced by the PS II acceptor side, such that a cycle around PS II is created. Interestingly, Schenck et al. [43] found the formation of the carotenoid radical cation inhibited by DCMU. Hence, in principle quenching at I₁ could be explained by a rapid cycle around PS II involving an intermediate carrier C, which could be a carotenoid or cyt b 559. This hypothetical cycle would cause a kind of “dissipative photochemical quenching”.

A common feature of all three tentative interpretations of the presented data is that the quenching at I₁ is caused by a limitation of the donation rate to P 680⁺. Whenever the life-time of P 680⁺ is long, it may either accumulate in strong continuous light, or may be re-reduced by recombination (i.e. direct backreaction) or by a cycle involving alternate donors, which oxidize the primary or secondary acceptors — all of which results in fluorescence quenching. In the case of ADRY reagents one should assume that only a rapid cycle around PS II induced by these substances can explain the quenching at the observed high donation rates to P 680⁺ [31]. We also notice that in all three cases, the quenching will affect the quantum yield of fluorescence to the same extent as that of PS II linear electron transport. At present, it is not possible to distinguish between these possibilities by measurements of fluorescence alone. Parallel measurements of P 680⁺, cyt b 559 and carotenoid absorption are required for a further analysis.

Finally, the question shall be addressed, in what way the presented data affect the validity of the quenching analysis by the saturation pulse method. In principle, this method can work only when full reduction of PS II acceptors during a saturation pulse is achieved [2–4]. Obviously, this cannot be guaranteed when the PS II donor side is limiting. From the presented results, we tend to conclude that in control samples the initial limitation of donation rate, expressed at I₁, is overcome during the first 50 msec of saturating illumination, expressed by the D-I₂ phase. With the rationale of the saturation pulse method [3, 4] this would be interpreted as non-photochemical fluorescence quenching. From the above discussion of possible interpretations of quenching at I₁, it is clear, that part of this “non-photochemical quenching” actually could be a type of “dissipative photochemical quenching” caused by rapid recombination or electron cycling at PS II centers. In this context, it is important to note that also the classical “energy-dependent” non-photochemical quenching, linked to internal acidification of the thylakoids (see ref. [1]), could be interpreted on the basis of a low-pH induced limitation of donation rate to PS II. As was shown by a number of researchers (see e.g. ref. [44, 45]), P 680⁺ reduction is considerably slowed down at low pH. This should lead to an increase of donor side dependent fluores-
cence quenching, in particular with that part of PS II centers which display already under control conditions a low donation rate (35 µs component; see discussion above). If this population indeed would increase upon internal acidification of the thylakoids [35], the donor side dependent quenching characterized in the present communication, may well be responsible for a substantial part of "energy-dependent" fluorescence quenching. In this context a recent finding of Oxborough and Horton [46] may be important that antimycin A prevents "energy dependent" fluorescence quenching, without affecting the internal acidification. Hence, the mechanism of "energy dependent" quenching appears to depend on a pH-dependent redox reaction, presumably involving a b-type cytochrome which may be O_{2} [37].

Recently, Weis et al. [47] concluded from saturation pulse quenching analysis of fluorescence, and comparative measurements of electron transport, that PS II quantum yield of open reaction centers is lowered by the same dissipative mechanism which causes "energy-dependent" fluorescence quenching. It was proposed that two populations of PS II exist, non-energized and energized, with the energized centers displaying almost no variable fluorescence yield and low photochemical yield (about 30% of normal yield). The excellent agreement between rates calculated from fluorescence quenching in intact leaves and rates measured by gas exchange under a variety of conditions [48] suggests that the Weis-model closely describes the in vivo state of PS II. The model does not give any mechanistic interpretation of "energy-dependent" quenching. It will be a challenging task for future work to investigate whether the donor-side dependent quenching characterized in our study could be the basis for the "energy control" of PS II quantum yield emerging from the work of Weis and co-workers [47, 48].

Acknowledgements

We wish to thank Wolfgang Bilger, Jan Snel and Ulrich Heber for fruitful discussions. Annette Weber and Ulrich Schliwa are thanked for technical assistance. We gratefully acknowledge financial support by the Deutsche Forschungsgemeinschaft (SFB 176 and La-54/32).