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Valinomycin sensitivity proves that light-induced thylakoid voltages result in millisecond phase of chlorophyll fluorescence transients

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Abstract

Upon sudden exposure of plants to an actinic light of saturating intensity, the yield of chlorophyll fluorescence increases typically by 200–400% of the initial O-level. At least three distinct phases of these O–J–I–P transients can be resolved: O–J (0.05–5 ms), J–I (5–50 ms), and I–P (50–1000 ms). In thylakoid membranes, the J–I increase accounts for ~ 30% of the total fluorescence increase; in Photosystem II membranes, the J–I phase is always lacking. In the presence of the ionophore valinomycin, which is known to inhibit specifically the formation of membrane voltages, the magnitude of the J–I phase is clearly diminished; in the presence of valinomycin supplemented by potassium, the J–I phase is fully suppressed. We conclude that the light-driven formation of the thylakoid-membrane voltage results in an increase of the chlorophyll excited-state lifetime, a phenomenon explainable by the electric-field-induced shift of the free-energy level of the primary radical pair [Dau and Sauer, Biochim. Biophys. Acta 1102 (1992) 91]. The assignment of the J–I increase in the fluorescence yield enhances the potential of using O–J–I–P fluorescence transients for investigations on photosynthesis in intact organisms. A putative role of thylakoid voltages in protection of PSII against photoinhibitory damage is discussed.

Keywords: Electric field effect; Fluorescence induction; Photosynthesis; Photosystem II; Variable fluorescence

1. Introduction

From an energetic point of view, life on earth is driven by photosynthetic solar energy conversion. In plants and cyanobacteria, the so-called light reactions take place in or at the thylakoid membrane, a partially stacked lipid bilayer membrane separating the lumen and the stroma space. The photosynthetic light reactions involve a series of electron transfer processes between protein–cofactor complexes and associated protonation events. Decades of multidisciplinary research have brought stunning insights in numerous details of the photosynthetic machinery. Some questions of fundamental importance (e.g., the mechanism of water oxidation) are still unresolved, but overall, a high level of understanding has been reached. Today, photosynthetic cofactor–protein complexes have become model systems to study the principles of biological electron and proton transfer processes at a molecular, atomic and electronic level. Taking into consideration the reached level of understanding of mechanistic details, we feel that it could be useful to supplement the atomic-level studies on isolated protein–cofactor complexes by new investigations on the role of the transmembrane electrical potential gradient (or the 'membrane voltage') as it is formed in photosynthetic organisms and intact thylakoid-membrane preparations. The magnitude of the thylakoid-membrane voltage is a factor, which could affect significantly energetics and interplay of the involved protein–cofactor complexes. In this work, we provide new evidence that there is a pronounced influence of the thylakoid voltage on the excited-state lifetime of antenna chlorophylls. On the basis of the proposed interpretation of so-called fluorescence transients, the influence of thylakoid voltages on the excited-state lifetime can be easily assessed not only in thylakoid preparations but also noninvasively in intact organisms.

The light-driven electron transport is coupled to ion fluxes across the thylakoid membrane resulting in the formation of an electric potential gradient ($\Delta\psi$), the thylakoid voltage, being positive at the lumenal side and negative at the stromal side [1–6]. Consequently, under continuous illumination, the charge-transfer reactions of the photosystems, PSII and PSI, have to proceed in the presence of an electric field

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opposing the vectorial electron transfer processes directed from the lumenal to the stromal side. Evidence has been presented that an electric field across the thylakoid membrane can affect the yield of the Chl fluorescence emitted by PSII [7–14]. The results of simultaneous measurements of electrochromic absorbance changes and light-induced changes in the Chl fluorescence yield are suggestive that the light-induced increase in $\Delta\psi$ is coupled to an increase in the Chl fluorescence yield [12]. Dau et al. [12] discussed this observation in the context of the reversible radical pair (RRP model [15], see also Refs. [16,17] and Fig. 1); they have proposed that electric field-induced changes of the charge-separation rate constants k_1, k_{-1} and k_2 are responsible for an increase in the Chl fluorescence yield.

Dau and Sauer [13,14] created a trans-thylakoid diffusion potential and, using time-resolved fluorescence spectroscopy, they investigated quantitatively the previously proposed mode of action of membrane voltages on the excited-state lifetime. They conclude that the membrane voltage results in an electric field which affects the energy of the [P680$^+$ Pheo$^-$] radical pair in its low-dielectric protein environment, a decrease in the free-energy difference, ΔG_0, between the excited chlorophyll-antenna state, [LHC + core + P680]*, and the charge separated state, [P680$^+$ Pheo$^-$], is observed. This decrease in ΔG_0 leads to a decrease in the rate constant of primary charge separation, k_1, and an increase in the rate constant of charge recombination, k_{-1}. Both, the slowed-down forward reaction (decrease in k_1) and the accelerated charge recombination (increase in k_{-1}) result in an increase of the Chl fluorescence yield.

The time course of the Chl fluorescence yield induced by exposure of plant leaves, algae, cyanobacteria, isolated chloroplasts, thylakoid membranes or PSII membrane particles to continuous illumination has been termed 'fluorescence induction curve' or 'fluorescence transient' [16,18–24]. Such fluorescence transients are widely used to investigate the photosynthetic light reactions in photosynthetic preparations. Perhaps more important, fluorescence transients also can be used to monitor various photosynthetic processes at a molecular level in intact organisms.

Upon exposure to the sudden high-intensity illumination, the fluorescence transients of leaves, algae, cyanobacteria, intact chloroplasts and intact thylakoid membranes are characterized by a polyphasic rise with the intermediate levels O, I_1, I_2 and P [25,26] or, alternatively, O, J, I and P [27]; in the following, the nomenclature of Strasser et al. [27] is used. The kinetics of the high-intensity fluorescence transients have been studied already 35 years ago by Delmose [28]. However, the interpretation of the various phases of these fluorescence transients is still a matter of debate. Mainly based on experiments using DCMU, it has been concluded that the O–J phase reflects reduction of the primary quinone electron acceptor Q_A [25,27–29]. Fluorescence rise from J to P level (passing the intermediate I level) is related to the reduction of the so-called plastoquinone (PQ) pool [25,27,29–32]. However, an unambiguous assignment is still lacking. This phase has been suggested to be related to heterogeneities: one rapidly and one slowly reducible PQ pool [27,33] or $PSII_\alpha/PSII_\beta$ heterogeneity [34]. Recent results point toward an alternative interpretation [32].

Already in the 1980s, features of Chl fluorescence transients have been hypothesized to be related to light-induced thylakoid voltages [35,36]; these hypotheses, however, remained tentative and unconfirmed. The light-induced thylakoid voltage is formed within 10–100 ms after onset of continuous actinic light [9,12,37–40]. A significant fluorescence increase occurring concomitantly with the formation of the thylakoid voltage should give rise to a resolvable kinetic phase of the fluorescence transient. Only the J–I increase of the fluorescence transient could match the kinetics of membrane-voltage formation. In this work, we provide evidence that the build-up of the thylakoid voltage is indeed responsible for the J–I rise of the Chl fluorescence transients.

2. Materials and methods

Thylakoid membranes were prepared from fresh market spinach (*Spinacia oleracea*); dim green light was used

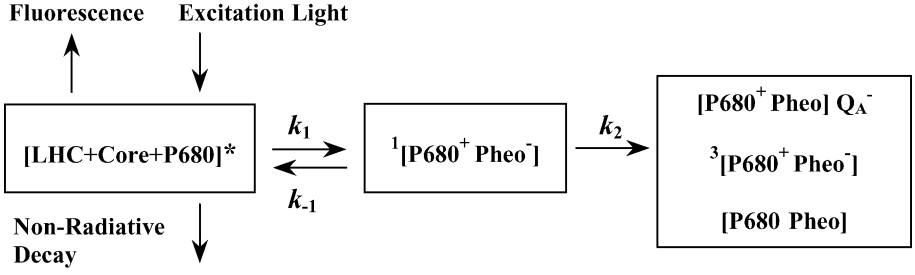

Fig. 1. Reversible radical pair model. Rapid excitation-energy transfer between the pigments of the LHC polypeptides and the PSII core antenna (including P680) is assumed to result in rapid establishment of an excited-state equilibrium distribution. The decay of this excited antenna state by formation of the primary radical pair is characterized by a (gross) rate constant denoted as k_1. The primary radical pair, [P680$^+$ Pheo$^-$], may decay by charge recombination resulting in repopulation the excited-antenna state (k_{-1}) or by one of the following processes: charge stabilisation resulting in reduction of Q_A, spin dephasing resulting in ^{3}[P680$^+$ Pheo$^-$] and nonradiative decays leading directly to the ground state. These three decay routes are assumed to be irreversible (to a first approximation); the sum of the respective rate constants is represented by k_2.

throughout the preparation. The spinach leaves were washed and quickly homogenized in the grind buffer containing 400 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM EDTA, 25 mM Hepes–NaOH (pH 7.5) (Medium A). After filtering through several layers of cheesecloth, the homogenate was centrifuged at 8900 × *g* for 10 min. The pellet was resuspended in a hypotonic buffer containing 150 mM NaCl, 5 mM $MgCl_2$, 25 mM Mes–NaOH (pH 6.2) (Medium B) and centrifuged again at 8900 × *g* for 10 min. The pellet was resuspended in 1 M glycine betaine, 15 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 25 mM Hepes–NaOH (pH 7.1) (Medium C) and centrifuged 1100 × *g* for 3 min. The supernatant was centrifuged at 48 400 × *g* for 5 min and the resulting pellet was resuspended in medium C. Immediately after preparation, the thylakoid membranes were used for measurements. PSII membrane particles were isolated from spinach (*S. oleracea*) using the Triton X-100 approach of Berthold et al. [41] with the significant modifications described in Schiller and Dau [42]. The PSII membrane particles were frozen at a chlorophyll concentration of about 4 mg/ml in a small aliquot and stored at − 80 °C until use.

For fluorescence measurements, freshly prepared thylakoid membranes were suspended in medium C. The PSII membrane particles were suspended in 1 M glycine betaine, 10 mM NaCl, 5 mM $CaCl_2$ and 25 mM Mes–NaOH (pH 6). A chlorophyll content of 1 μg Chl/ml was used for all fluorescence measurements. For some measurements, valinomycin or nigericin previously dissolved in DMSO were added to yield a final ionophore concentration of 1 μM. The final concentration of DMSO in the measuring solution always stayed below 1%.

Chlorophyll fluorescence transients induced by high light intensity (3500 μmol m^{-2} s^{-1}) were measured using the laboratory-built LED-Array fluorometer described in Pospíšil and Dau [32]. The magnitude of the (small) offset resulting from scattered light was estimated by measurements on pure buffer and subtracted from the fluorescence signal. Data processing and curve fitting were performed using EXCEL (Microsoft, USA). The *R*-factor providing the mean deviation between calculated and experimental values in percentage was calculated using Eq. (1).

$$R = \frac{\sqrt{\frac{1}{N}\sum_{i=1}^{N}(F_i^{\exp} - F_i^{\sim})^2}}{\frac{1}{N}\sum_{I=1}^{N}|F_i^{\exp}|}\,100\% \qquad (1)$$

3. Results

In both thylakoid membranes and PSII membrane particles, the onset of the saturating actinic light results in an increase of the fluorescence from the O-level (corresponding to F_0, the minimal fluorescence yield) to the J-level; the J-level is reached within 2–10 ms after onset of illumination (Fig. 2). In thylakoid membranes, the O–J phase is followed by an increase to the I-level which is reached at 20–100 ms. The J–I phase is followed by an increase to the P-level (corresponds to F_M, the maximal fluorescence yield). The final increase to the maximal fluorescence yield is completed within about 1 s. In clear contrast to the thylakoid preparation, in PSII membrane particles, the O–J phase is followed directly by a slow mono-exponential increase to the P-level; the intermediate J–I phase is lacking.

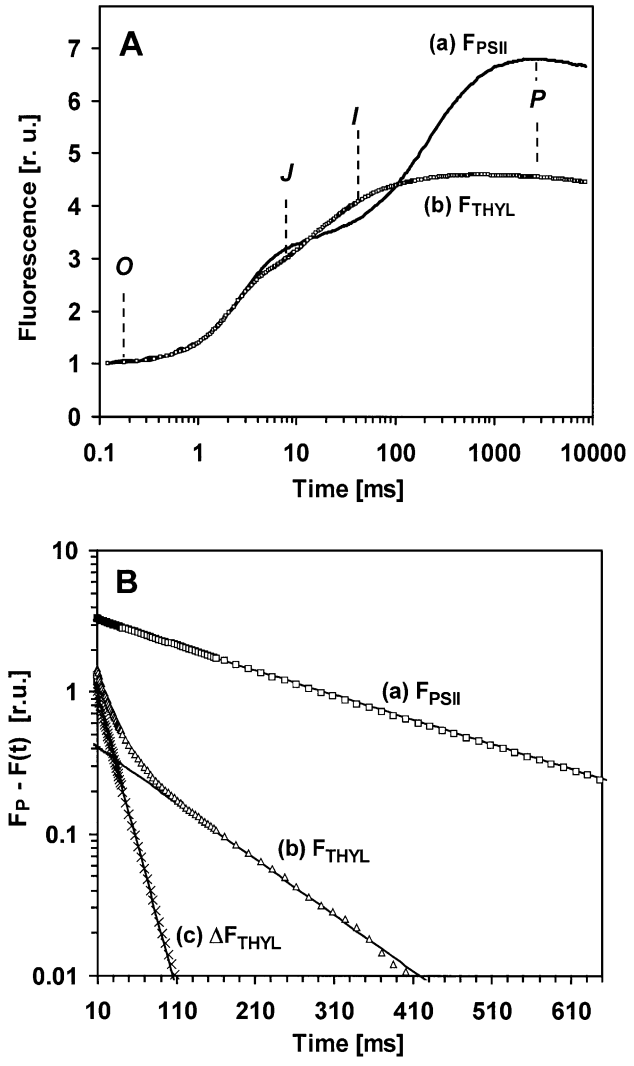

Fig. 2. Chlorophyll fluorescence transients of (a) PSII membrane particles and (b) thylakoid membranes. The Chl fluorescence yield at O-level was normalized to unity. In B, for times ranging from 10 to 650 ms, the difference between the peak level, F_P, and the actual fluorescence level, $F(t)$, is shown using a logarithmic *y*-axis. In B, straight (solid) lines are shown that fit the data points of (a) PSII membrane particles for times ranging from 10 to 650 ms and of (b) thylakoids for time ranging from 120 to 400 ms. For times ranging from 10 to 110 ms, deviations between the straight line (solid line) and the data (triangles) are visible only for thylakoids. These deviations are plotted in B [curve (c)]. The three straight solid lines in B represent exponential time dependencies; their slopes correspond to time constants of 240 (a), 110 (b), and 21 ms (c).

In Fig. 2B, the difference of the actual fluorescence level [$F(t)$] to the peak level (F_P) is plotted for times ranging from 10 to 650 ms. Using a logarithmic y-axis, for PSII membrane particles, a straight line is obtained demonstrating that in PSII membrane particles, the increase to the peak level is perfectly well described as a slow mono-exponential rise (halftime greater than 100 ms). In clear contrast, in thylakoids, the corresponding fluorescence increase is clearly bi-exponential [see curves (b) and (c) in Fig. 2B] with a 20-ms component (J–I phase) and a 100-ms component (I–P phase). These results of the graphical analysis are confirmed by numerical simulations (curve fitting) presented further below. In conclusion, the J–I phase is clearly resolvable in thylakoid preparations, but completely lacking in PSII membrane particles.

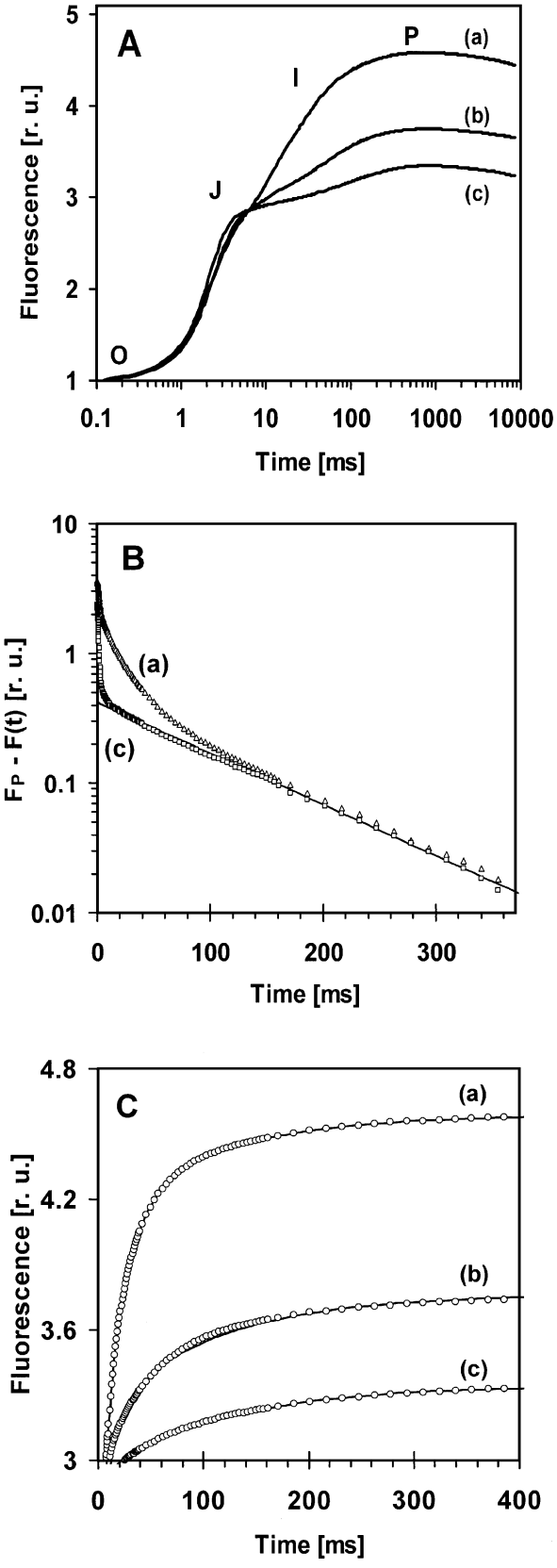

Fig. 3. Effect of valinomycin on fluorescence transients of thylakoid membranes: (a) control, (b) 1 μM valinomycin, (c) 1 μM valinomycin supported by 5 mM KCl. The Chl fluorescence yield at O-level was normalized to unity. (A) Full-time courses using a logarithmic time scale. (B) Difference between the peak level, F_P, and the actual fluorescence level, $F(t)$, using a logarithmic y-axis, but linear time axis. (C) Data points (open circles) and simulations (solid lines) using three exponential functions (Eq. (2)). The corresponding simulation parameters are listed in Table 1.

The O–J rise observed in the thylakoid membranes results from the reduction of Q_A [25,29]. Recently, it has been demonstrated that in the presence of DCMU, which inhibits the Q_A–Q_B electron-transfer, the J–P phase is absent not only in thylakoid membranes, but also in PSII membrane particles [32]. Thus, we have concluded that, in analogy to the situation encountered in thylakoid membranes, the O–J phase in PSII membranes is explainable by the reduction of Q_A and the concomitant increase in the fluorescence yield. The subsequent J–P rise has been proposed to result from reduction of the pool of mobile plastoquinone molecules present in the lipid bilayer membrane of PSII membrane particles [32]. The oxidized form of PQ molecule serves as fluorescence quencher, whereas the reduced form is unable to quench fluorescence [43]. Therefore, the reduction of oxidized PQ molecule results in the removal of fluorescence quenching and thus in the J–P rise. The J–P rise is always clearly more pronounced in PSII membrane particles than in thylakoid membranes. This observation is explainable by differences in the PQ quenching efficiency [32,44].

The lack of the intermediate I-level in the PSII membranes is of particular interest. In the thylakoid preparation, the largely intact membrane separates an inner compartment, the lumen, and an outer compartment, the stroma space. Consequently, light-induced electron transfer across the thylakoid membrane and associated ion translocations result in formation of an electric potential difference, $\Delta\psi$, between lumen and stroma space. This thylakoid-membrane voltage is formed within a few milliseconds after onset of illumination [9,12,37–40]. In PSII membrane particles, however, the PSII is embedded in membrane sheets not separating compartments; consequently, formation of a membrane voltage is impossible. Thus, absence of the transmembrane voltage in the PSII preparation could be related to the lack of the intermediate I-level.

To elucidate further the role of membrane voltages, we measured chlorophyll fluorescence transients of thylakoid membranes in the presence of ionophores. Valinomycin, a potassium-specific ionophore, facilitates translocation of potassium ions (and with lower rates of other cations) across the thylakoid membrane resulting in a 'short-circuit' with respect to the membrane voltage. In the presence of potassium, valinomycin specifically inhibits formation of the transmembrane voltage. The influence of valinomycin on the chlorophyll-fluorescence transients is shown in Fig. 3. All fluorescence transients measured for valinomycin-treated thylakoid membranes in a standard measuring medium exhibit partial suppression of the J–I phase (Fig. 3, trace b); in media supplemented with potassium chloride, the J–I phase seems to be fully absent (Fig. 3, trace c; see also Table 1).

To quantify the effect of valinomycin on the J–I phase, fluorescence transients were simulated using a sum of three exponential functions:

$$F(t) = F_O + A_{O-J}(1 - e^{-t/\tau_{O-J}}) + A_{J-I}(1 - e^{-t/\tau_{J-I}}) + A_{I-P}(1 - e^{-t/\tau_{I-P}}) \quad (2)$$

where F_0 is the minimal fluorescence; A_{O-J}, A_{J-I} and A_{I-P} are amplitudes; τ_{O-J}, τ_{J-I} and τ_{I-P} are time constants of the O–J, J–I and I–P phase, respectively. The tri-exponential model facilitates an essentially perfect description of the fluorescence transients in the time-range of interest. In Fig. 3B, the lines represent the best fit to the experimental data (open circles); the resulting fit parameters are shown in Table 1. The observation that the amplitudes of O–J and I–P phases remain relatively unchanged upon valinomycin addition indicates that there is no major effect of the thylakoid voltage on the processes associated with the O–J and I–P phases. In contrast, the amplitude of the J–I phase decreases by 75% when valinomycin is present and reaches zero if the action of the $\Delta\psi$-dissipating valinomycin is supported by KCl.

Table 1
Influence of valinomycin on amplitudes and time constants of O–J, J–I and I–P phases of the fluorescence transients of thylakoid membranes shown in Fig. 2

	$F_O + A_{O-J}$	A_{J-I}	τ_{J-I} [ms]	A_{I-P}	τ_{I-P} [ms]	R [%]
thylakoid	2.6	1.5	21	0.46	110	0.02
thylakoid + valinomycin	2.8	0.4	21*	0.55	110*	0.44
thylakoid + valinomycin + K^+	2.9	0*	–	0.41	115	0.32
PSII particles	3.2	0*	–	3.43	244	0.09
thylakoid + nigericin	2.2	1.3	21*	0.46	110*	0.39

The amplitudes and time constants obtained for PSII membrane particles (second last row) and for the nigericin control (thylakoid membranes plus nigericin, last row) are also listed. Parameters obtained by an exponential fit according to Eq. (2) are shown (τ_{O-J} = 3.5 ms in all simulations). The value of R represents the average deviation between calculated values and data points in percentage for times ranging from 10 to 400 ms (Eq. (1)). Values labelled by * were not varied in the course of the curve fitting (fixed values).

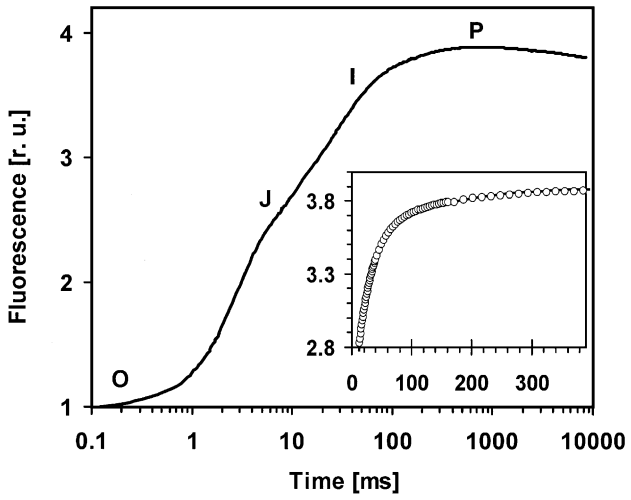

Fig. 4. Unchanged OJIP transients of thylakoid membranes treated with the protonophore nigericin (1 μM). The inset shows data points (circles) and simulation results (solid line) for the time period ranging from 10–390 ms using a linear time scale. The corresponding simulation parameters are listed in Table 1.

The partial suppression of the J–I phase in the absence of added potassium is explainable either by residual potassium ions in the preparation or by translocation of sodium ions. It has been demonstrated that valinomycin facilitates not only potassium, but also sodium translocation [45,46]. However, since the binding affinity of valinomycin towards sodium is relatively low, formation of a membrane-voltage formation is only partially inhibited in the absence of potassium [45,47].

The influence of valinomycin and its relation to the potassium concentrations is highly suggestive of a close relation between the J–I phase and membrane voltages. To confirm that the suppression of the J–I phase is specifically related to the $\Delta\psi$-dissipating effect of valinomycin, the effect of another ionophore, namely nigericin, was studied. In presence of nigericin, which catalyzes electrically neutral exchange of K^+ and H^+ cations and thus prevents the creation of ΔpH without affecting $\Delta\psi$, the typical triphasic OJIP pattern with no suppression of the J–I phase is observed (Fig. 4, Table 1).

4. Discussion

In bioenergetics, phenomena related to the formation of membrane voltages are frequently identified by their valinomycin sensitivity. Visual inspection of Fig. 3 and the simulation results presented in Table 1 show that specifically, the J–I increase (but not the I–P increase) is inhibited by valinomycin. Therefore, we conclude that the so-called J–I phase (of the light-induced increase in the yield of Chl fluorescence) is closely related to the electrical potential gradient, $\Delta\Psi$, across the thylakoid membrane. The assignment of the J–I increase in the fluorescence yield to $\Delta\Psi$-

formation also provides a straightforward explanation for the absence of this phase in fluorescence transients of PSII membrane particles which lack a closed lumen compartment.

It has been reported that valinomycin (supplemented by potassium) does not only prevent formation of the thylakoid voltage, but also may affect the cytochrome *b*-563 reduction directly (meaning not via the membrane potential). Specifically, in the presence of valinomycin, the rate constant for cytochrome *b*-563 reduction has been found to be reduced (by less than 50%) [48,49]. This valinomycin effect seems to be unrelated to the here observed effect on the PSII fluorescence. If the J–I increase reflected directly the reduction of the cytochrome *b*-563, we should observe a moderate valinomycin-induced decrease in the corresponding rate constant. Instead, we observe unchanged rate constants and a complete disappearance of the J–I rise. Furthermore, due to the presence of several PQ molecules (the so-called PQ pool), the direct 'translation' of the valinomycin effect on the cytochrome *b*/*f* complex into an effect on the PSII fluorescence would require an interaction between the PSII complex and the cytochrome *b*/*f* complex not mediated by the molecules of the PQ pool. The existence of such an interaction that bypasses the PQ-pool is unlikely.

In general, fluorescence induction curves do not reflect directly electron transfer processes occurring at the cytochrome *b*/*f* complex or around PSI, because the electron translocation from PSII to the cytochrome *b*/*f* complex is mediated by a 'pool' of several mobile PQ molecules. This PQ-pool acts as a 'kinetic buffer' thus preventing that the rate constants of the relatively fast processes involving the cytochrome *b*/*f* complex, PSI, or the PS I donor side are directly 'visible' in the kinetics of the PSII fluorescence (see, e.g., Refs. [19,50]). However, inhibition (or acceleration) of processes at the cytochrome *b*/*f* complex (or also at PSI) might affect the OJIP transients indirectly by influencing the gross rate of the PQ-pool reduction. Because valinomycin does not influence the rate of the I–P fluorescence rise, which is assignable to the reduction of the PQ-pool, such an indirect valinomycin effect on the OJIP transients is unlikely to be of relevance.

Using intact leaves, Dau et al. [12] compared light-induced changes in the fluorescence yield, Y_F, and the electrochromic absorbance changes at 518 nm, ΔA_{518}, which are commonly assumed to be indicative of $\Delta\Psi$-formation (at least in the millisecond range). By means of a detailed kinetic analysis of the simultaneously measured ΔY_F and ΔA_{518}, they have shown that an increase in the fluorescence yield occurs concomitantly to the light-induced formation of ΔA_{518}. The observed kinetic coincidence, however, might have been fortuitous; proving the valinomycin sensitivity has not been approached (because the use of uncouplers is problematic in intact leaves). In the present work, by analysis of OJIP transients of thylakoid preparations, proof is provided that a millisecond phase of the light-induced fluorescence increase is indeed valinomycin sensitive. Thus, we can safely conclude that in intact organisms and preparations of (intact) thylakoid membranes, the light-induced formation of a transmembrane voltage results in a pronounced increase in the fluorescence yield. Mechanistically, this $\Delta\Psi$-dependent increase in the fluorescence yield is explainable by an electric-field effect on the rate constants of primary and secondary charge separation in PSII (see Introduction).

The magnitude of the voltage-dependent increase in the excited-state lifetime (increase by about 50%) is remarkable. In intact photosynthetic organisms, irradiation with light of high intensity causes an only slowly reversible drop of photosynthetic performance (photoinhibition), which is related to processes damaging the PSII (for reviews, see: Refs. [51–54]). Seemingly, photodamage to PSII constitutes a major 'problem' to plants and cyanobacteria; suitable regulatory and protective mechanisms provide a significant evolutionary advantage. Photosynthetic organisms are equipped with a variety of protective mechanisms to minimize the detrimental influence of (excessive) light (for reviews, see: Refs. [19,55–57]). As outlined in the following, also the effect of light-induced membrane voltages on charge separation reactions and excited-state lifetime may contribute to the protection of PSII.

The electric field resulting from light-induced thylakoid voltages necessarily shifts the free-energy difference between the primary-radical pair state and the singlet-state energy of chlorophylls. Consequently, the mean population time of the radical-pair state decreases and the excited-state lifetime increases (see Introduction). Upon exposure of the photosynthetic organism to high-intensity illumination, the primary quinone acceptor, Q_A, is in its singly reduced state in numerous PSII. Therefore, the probability for decay of the primary radical pair by processes which eventually can damage PSII is increased. (These processes are: formation of Chl triplets by the radical-pair-recombination mechanism, double-reduction of Q_A, and detrimental oxidation reactions involving $P680^+$ [58–62].) If an electric field resulting from the light-induced thylakoid voltage decreases, the mean radical-pair population time, the probability of detrimental side reactions will become significantly reduced. Thus, the light-induced membrane voltage may play a role in protecting PSII against photodamage.

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