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Fluorescence decline as a function of redox potential and actinic light intensity in spinach thylakoids

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Excitation of isolated thylakoids with sufficiently strong actinic light increases the fluorescence quantum yield up to a maximum level, F_{max}, followed by a slower decline under certain experimental conditions. In this study the latter effect was analyzed as a function of the ambient redox potential and the actinic light intensity. Two different types of fluorescence decrease were found. (a) In the presence of specific quinones widely used as redox mediators a fast and comparatively small decrease (30% of F_{max}), referred to as ΔF_{SQ}, was observed at moderate redox potentials ($-300 < E_m < +200$ mV). ΔF_{SQ} disappears at positive values with $E_{m,7.5} = +110$ mV, whereas the decrease at negative redox potential depends on the midpoint potential of the quinone. (b) A more pronounced fluorescence decline was observed at redox potentials below -300 mV, which comprises 65–70% of the maximum fluorescence. The full expression of this effect, referred to as ΔF_{LP}^{max}, requires markedly higher actinic light intensities than ΔF_{SQ}^{max}. The extent of ΔF_{LP}^{max} as a function of the redox potential is dependent on the presence of redox mediators. In their absence the full expression of ΔF_{LP}^{max} can be only observed below -400 mV. Based on the hypothesis of Pheo$^-$ photoaccumulation being responsible for the fluorescence decline at low redox potentials (Klimov, V.V., Klevanik, A.V. and Shuvalov, V.A. (1977) FEBS Lett. 82, 182–186), a reaction scheme is presented that qualitatively describes the time course of ΔF_{LP} at different actinic light intensities and redox potentials. Based on this analysis, the rate of Pheo$^-$ reoxidation is inferred to be limited by the reaction center apoprotein acting as a barrier to redox equilibration. The implications for the interpretations of redox titration curves are briefly discussed.

Introduction

Photosynthetic water cleavage by visible light is initiated by exciton dissociation within the reaction center complex of Photosystem II. This complex contains a special chlorophyll *a* (referred to as P-680) as the photoactive component that transfers an electron from its excited singlet state to an intermediary redox group I, which has been identified as pheophytin *a* (for review, see Ref. 1). A stabilization of the primary charge separation sufficient for water cleavage can be only achieved if further electron transfer is possible from I$^-$ to the primary plastoquinone acceptor, Q_A [2]. Indirect information about the free energy transformation of excitons into an electrochemical potential difference at the reaction center complex of PS II can be obtained by different fluorometric methods. It was shown that among different factors the fluorescence quantum yield strongly depends on the redox state of P-680, I and Q_A: P-680$^+$, I$^-$ and Q_A are quenching states with the π-cation/anion

Abbreviation: PS, Photosystem.

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radicals ($P\text{-}680^+$, I^-) acting as nonphotochemical quenchers, whereas Q_A is a photchemical quencher (for latest reviews, see Ref. 3). Under the frequently used conditions for measuring fluorescence induction curves, the very rapid transients of $P\text{-}680^+$ and I^- do not affect the increase of fluorescence yield. If, however, thylakoids or PS II preparations are illuminated with strong actinic light in the presence of $Na_2S_2O_4$, keeping Q_A^- chemically reduced, a comparatively slow fluorescence decline is observed that kinetically coincides with the photoaccumulation of $Pheo^-$ [4–7]. This effect raised the question whether or not the reduction of Q_A^- is a satisfying condition for $Pheo^-$ photoaccumulation. Keeping Q_A^- reduced in the presence of DCMU and the most powerful ADRY agent [8] 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene (ANT 2p) is not sufficient for a significant $Pheo^-$ photoaccumulation [9]. On the other hand, a comparatively rapid fluorescence decline was observed at moderate redox potentials if chloroplasts were illuminated in the presence of special quinoens [10]. However, as this effect was found to be markedly dependent on the type of compound the observed fluorescence decline is not easily explainable in terms of redox state changes of Pheo.

In order to analyze the different types of fluorescence decline of F_{max} induced by actinic light this phenomenon was studied as a function of light intensity and the redox potential of the suspension. Based on the finding that at sufficiently low redox potentials (below -300 mV) the fluorescence decline is dominated by the photoaccumulation of $Pheo^-$ [4–7], the kinetics observed at different light intensities were analyzed in terms of the most simple scheme for the PS II reaction center complex.

Materials and Methods

The experiments were performed with spinach chloroplasts (class II) prepared according to the method described by Winget et al. [11], except that 10 mM ascorbate was present in the grinding medium.

The experiments at different redox potentials were carried out under anaerobic conditions. The redox equilibration was performed in a reaction vessel under N_2 atmosphere at 15 °C in a solution containing chloroplasts (5 μM chlorophyll), 2 mM $MgCl_2$, 2 mM NaCl, 50 mM Tricine-NaOH (pH = 7.5) and the following redox mediators: 30 μM 2-hydroxy-1,4-naphtoquinone ($E_{m,7} = -145$ mV), 10 μM duroquinone ($E_{m,7} = 5$ mV) and 50 μM anthraquinone-2-sulfonic acid ($E_{m,7} = -225$ mV). Adjustment of the redox potential was achieved by additions of small aliquots of $K_3[Fe(CN)_6]$ or $Na_2S_2O_4$. For redox potential measurements a combined platinum-calomel electrode (Schott P65) and a Knick-pH-voltmeter were used.

The dark-incubation time, t_{inc}, before starting the measurement should be long enough to permit a sufficient redox equilibration, but on the other hand as short as possible in order to prevent deleterious effects on the activity. To optimize this parameter experiments were performed at low redox potentials. The data depicted in Fig. 1 indicate that a time of $t_{inc} = 30$ min properly satisfies the above-mentioned condition. After this dark incubation a small probe of the solution (1 ml) was transferred under N_2 atmosphere into the cuvette for fluorescence measurements. The fluorescence was measured from the top of the cuvette by a set-up similar to that described by Voss et al. [12]. The actinic light of a He-Ne laser ($\lambda = 632.8$ nm) was simultaneously used for exciting the fluorescence. The numerical calculations were performed by using a computer program resembling that described by Renger and Schulze [13].

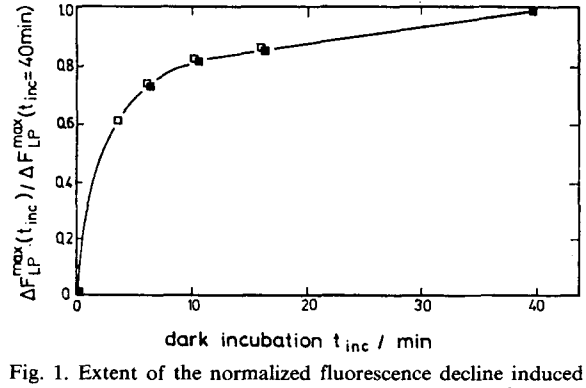

Fig. 1. Extent of the normalized fluorescence decline induced in isolated thylakoids by strong actinic light (43 W/m²) at pH 7.5 and a redox potential of −405 mV as a function of dark incubation time, t_{inc}. Open symbols represent measurements in the absence, closed symbols in the presence of the above-mentioned redox mediators.

Results

Fig. 2 shows typical traces of fluorescence induction curves at two different actinic light intensities, redox potentials and time scales, respectively. At moderate light intensities (7.7 W/m^2) and redox potentials above −300 mM a comparatively rapid fluorescence decline is observed that indispensably requires certain types of quinones and comprises about 30% of the maximum level F_{max} (the very fast fluorescence rise from F_0 to F_{max} due to Q_A^- reduction after switching on the actinic light cannot be resolved under the conditions of Fig. 2). The maximal extent of this effect, attained after a few seconds and referred to as ΔF_{SQ}^{max}, strongly depends on the redox potential of the suspension and of the specific quinones used, as is shown in Fig. 3. Complete removal of oxygen by the system glucose/glucose oxidase does not affect ΔF_{SQ}^{max}. Furthermore, the phenomenon is invariant to addition of gramicidin (30 nM) or NH_2OH (1.7 mM) or the omission of Mg^{2+} (data not shown). This indicates that a formation of a transmembrane electrochemical potential difference is not responsible for the ΔF_{SQ} effect. At higher actinic light intensities (43 W/m^2) the effect is less pronounced and, in addition, a transient increase is observed (see Fig. 2, A and B, top) which can be eliminated by gramicidin (see Fig. 1B, top, dotted lines). In agreement with previous conclusions [10], the ΔF_{SQ} phenomenon is interpreted as a redox reaction of special quinones with the PS II acceptor side because the decline of ΔF_{SQ}^{max} at negative redox potentials (see Fig. 3) exhibits an $E_{m,7.5}$ very similar to that of the corresponding quinones, anthraquinone-2-sulfonic acid ($E_{m,7.5} = -255$ mV) and 2-hydroxy-1,4-naphthoquinone ($E_{m,7.5} = -175$mV). It will not be further analyzed in this study.

At sufficiently negative redox potentials (−380 mM in Fig. 1) a completely different pattern arises. In this case a fluorescence decline is induced that requires markedly higher intensities and illumina-

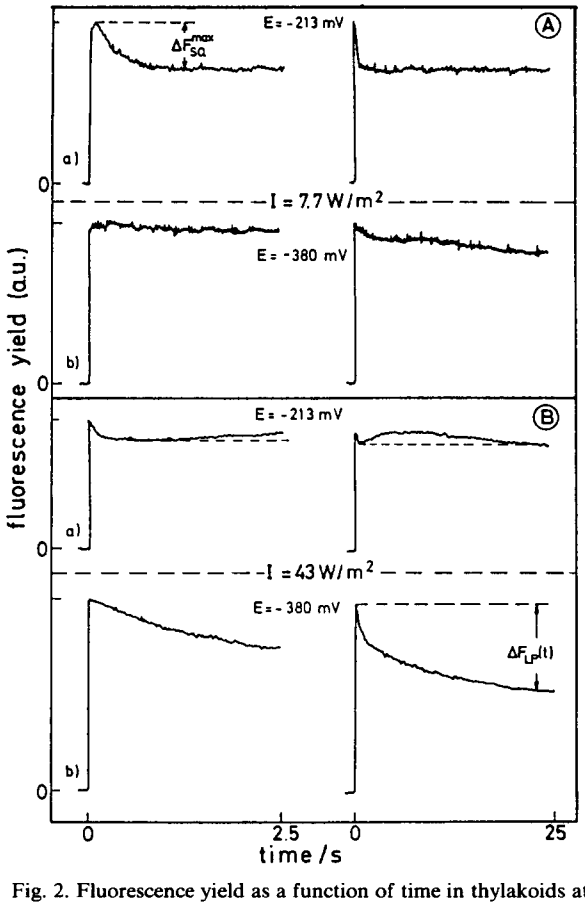

Fig. 2. Fluorescence yield as a function of time in thylakoids at pH 7.5 under strong CW illumination at different ambient redox potentials and actinic light intensity, respectively. The symbols ΔF_{SQ}^{max} and $\Delta F_{LP}(t)$ for the different types of fluorescence decline are explained in the text. The dotted curves in (B) top, were obtained in the presence of 45 nM gramicidin.

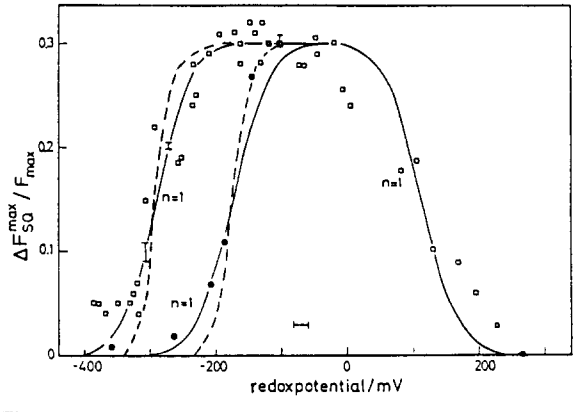

Fig. 3. Normalized fluorescence decline $\Delta F_{SQ}^{max}/F_{max}$ as a function of redox potential in thylakoids at pH 7.5. Actinic light intensity: 6 W/m^2. □, Addition of redox mediators. ●, The same as □, but without anthraquinone-2-sulfonic acid. The curves represent Nernst curves with $n = 1$ and $E_{m,7.5} = +110$ mV for the decline at positive redox potentials and $E_{m,7.5} = -290$ mV ($n = 1$, full line, $n = 2$, dotted curve) and −170 mV ($n = 1$ full line, $n = 2$, dotted curve).

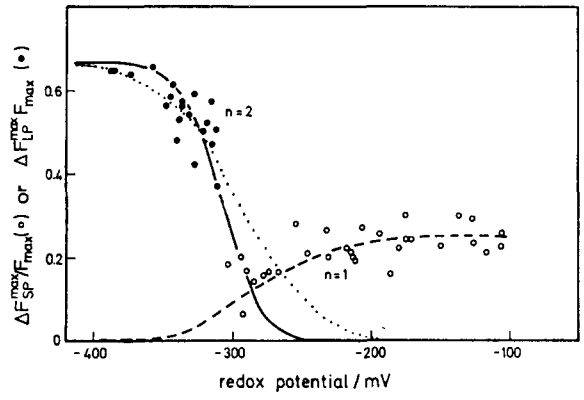

Fig. 4. Normalized fluorescence decline $\Delta F_{SQ}^{max}/F_{max}$ (○) and $\Delta F_{LP}^{max}/F_{max}$ (●) as a function of redox potential in thylakoids at pH 7.5 in presence of redox mediators. Actinic light intensity, 43 W/m^2. The curves were calculated with the following parameters: - - - - - -, $E_{m,7.5} = -280$ mV, $n = 1$; · · · · · ·, $E_{m,7.5} = -290$ mV, $n = 1$; ———, $E_{m,7.5} = -310$ mV, $n = 2$.

tion times (2–3 min) of actinic light to become fully expressed and the maximum extent referred to as ΔF_{LP}^{max} is significantly larger (up to 70% of F_{max}) than the above-mentioned ΔF_{SQ}^{max} effect. At sufficiently high actinic light intensities (vide infra, Fig. 7) ΔF_{LP}^{max} almost reaches the extent of the variable fluorescence of the fast rise from F_0 to F_{max}, comprising 75–80% of F_{max}. The fluorescence decline at low redox potentials $\Delta F_{LP}(t)$ was shown to be caused by Pheo$^-$ photoaccumulation [7–10].

Accordingly, ΔF_{LP}^{max} as a function of the redox potential should reflect the thermodynamic constraints of Pheo$^-$ photoaccumulation. Experiments were performed with strong actinic light. In Fig. 4 the normalized fluorescence yield decline $\Delta F^{max}/F_{max}$ is depicted as a function of the ambient redox potential. Two different types of phenomenon are observed. At redox potentials below -310 mV the above-mentioned ΔF_{LP} effect arises, whereas at redox potentials above -290 mV the shape of the fluorescence decline markedly changes, as is shown in Fig. 5. This might suggest that different mechanisms are responsible for the decrease of the fluorescence yield, and therefore different symbols were used to characterize these data. The closed circles are obtained from curves which exhibit the typical characteristics of a $\Delta F_{LP}(t)$ decline (see Fig. 2B, bottom and Fig. 5, right side). These data are assumed to reflect Pheo$^-$ photoaccumulation. On the other hand, the data symbolized by open circles could be related to the effect due to special quinones, ΔF_{SQ}^{max}. This idea is supported by the finding that their dependence on the redox potential can be roughly described by the Nernst curve derived for the decline of ΔF_{SQ}^{max} in Fig. 3. Around -300 mV a clear separation cannot be achieved between contribu-

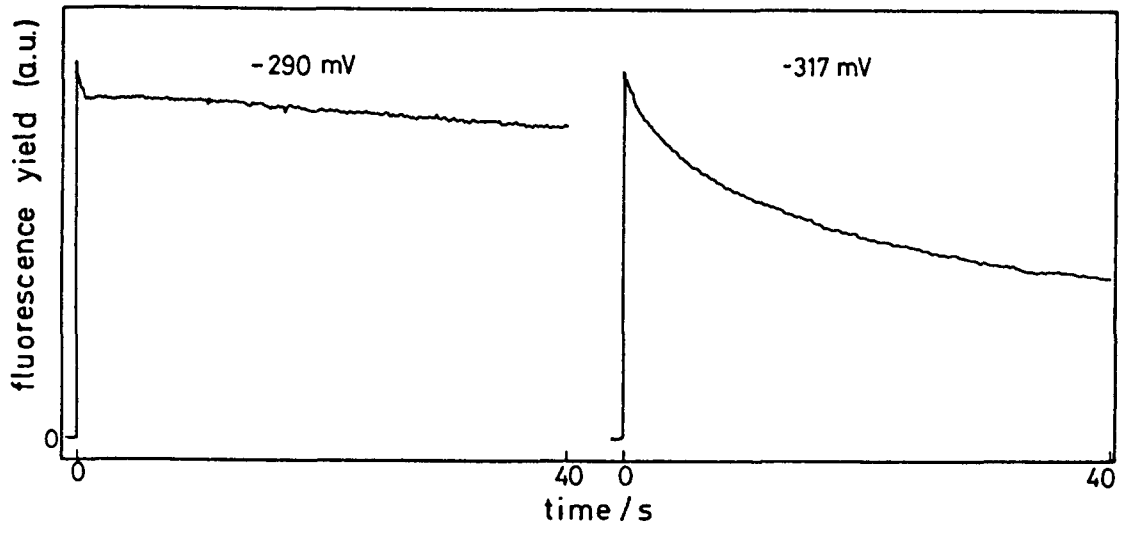

Fig. 5. Fluorescence yield as a function of time under strong CW illumination ($I = 43$ W/m^2) at redox potentials of -290 and -317 mV, respectively.

tions from ΔF_{SQ}^{max} and ΔF_{LP}^{max}. If the data of this small range are omitted the dependence of ΔF_{LP}^{max} on the redox potential appears to follow a two-electron ($n = 2$, full line) titration with a midpoint potential of $E_{m,7.5} = -310$ mV rather than a one-electron ($n = 1$, dotted line) process. Regardless of this detail, the question arises about the origin of the steep decrease of ΔF_{LP}^{max} around -300 mV. The light-induced decline of the fluorescence yield at low redox potential was shown to be due to photoaccumulation of the pheophytin anion radical $Pheo^{\bar{}}$ acting as nonphotochemical quencher [4–7]. Accordingly, the dependence of ΔF_{LP}^{max} on the redox potential is related to thermodynamic constraints of the formation of state P-680$Pheo^{\bar{}}$ $Q_A^{\bar{}}$. The kinetics of $Pheo^{\bar{}}$ photoaccumulation depend on actinic light intensity and on the photochemical quantum yield of the reaction, which is determined by the rate constants of P-680^{+} reduction and $Pheo^{\bar{}}$ reoxidation via radical pair recombination (P-680^{+} $Pheo^{\bar{}}Q_A^{\bar{}} \rightarrow$ P-680Pheo$Q_A^{\bar{}}$) and other components, respectively. Based on the latest findings, the kinetics of the back reaction appears to be almost invariant to redox potential down to -400 mV [14]. The kinetics of P-680^{+} reduction by Z strongly depend on the functional integrity of the water-oxidizing enzyme system [15–17]. In order to check possible effects of our experimental conditions comparative measurements were performed with untreated thylakoids in the absence and presence of hydroxylamine and with Tris-washed thylakoids. Surprisingly, no change was observed of the light-induced decline kinetics of the fluorescence yield $\Delta F_{LP}(t)/F_{max}$ in the different samples. This result suggests that an incubation with $Na_2S_2O_4$ affects the water-oxidizing enzyme system. Direct measurements of the average oxygen yield per flash confirm that in thylakoids incubated in the dark with $Na_2S_2O_4$ (30 min) followed by centrifugation and resuspension in buffer without $Na_2S_2O_4$ the oxygen evolution capacity becomes drastically diminished. This effect is not observed if the same procedure is performed in the absence of $Na_2S_2O_4$ (Renger, G., Fromme, R., Eckert, H.-J. and Kayed, A, unpublished results). Accordingly, a functionally competent water oxidizing enzyme system is not a prerequisite for the fluorescence decline $\Delta F_{LP}(t)$ due to $Pheo^{\bar{}}$ photoaccumulation. This result is not in line with previous conclusions [18]. The implication of this finding will not be analyzed further in this study. In samples deprived of their oxygen evolution capacity the reduction of P-680^{+} by Z is dominated by pH-dependent kinetics with half-lifetimes of 2–20 μs [16]. These kinetics, which were found to be resistant to rather harsh trypsin treatments of PS-II membrane fragments [19], are rate-limiting for P680^{+} reduction under our experimental conditions of determining ΔF_{LP}^{max}. P-680^{+} reduction by Z is expected to be independent of the redox potential in the range analyzed in this study. Therefore, the sharp decrease of ΔF_{LP}^{max} in -350 mV $< E_m <$ -290 mV at constant actinic light intensity (see Fig. 4) reflects an increase of the overall rate of $Pheo^{\bar{}}$ reoxidation by components other than P-680^{+} (radical pair recombination or $Q_A^{\bar{}}$ (kept reduced). This reaction will be characterized by an overall rate constant symbolized by k_{Pheo}. First, the reoxidation of $Pheo^{\bar{}}$ in the dark was estimated from the recovery of the high fluorescence yield after actinic illumination, giving rise to ΔF_{LP}^{max} at -405 mV. In Fig. 6 the ratio of ΔF_{LP}^{max} due to a second illumination ΔF_{LP}^{max} (2nd ill., t_d) normalized to that caused by the first illumination of 160 s is depicted as a function of the dark time between both illuminations. The recovery kinetics exhibit a biphasicity with half-rise times of about 0.5 and 10 min, respectively. This biphasicity could be indicative for a heterogeneity in the population of PS II reaction centers. Another interesting finding is the observation that the decline of the fluorescence yield $\Delta F_{LP}(t)$ is faster

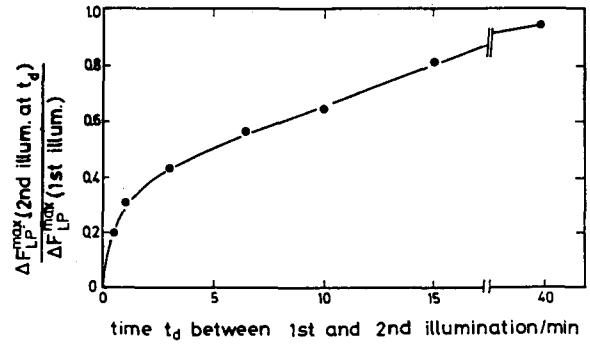

Fig. 6. Extent of the normalized decay ΔF_{LP}^{max} as a function of the dark time t_d after the first actinic light exposure of thylakoids at pH 7.5 and $E = -405$ mV. Actinic light intensity, 43 W/m^2.

during the second illumination (data not shown). This phenomenon is assumed to reflect modifications of the reaction pattern in PS II due to strong actinic light in the presence of $Na_2S_2O_4$. Independent lines of evidence obtained from flash-induced absorption changes and fluorescence life time measurements confirm this conclusion [14].

In the simplest case the time-course of $\Delta F_{LP}(t)$ and its maximum extent ΔF_{LP}^{max} are dependent on the actinic light intensity and the overall rate of $Pheo^-$ reoxidation characterized by a rate constant k_{Pheo}. Accordingly, at a definite redox potential the time course of $\Delta F_{LP}(t)$ and the maximal extent ΔF_{LP}^{max} should only depend on the actinic light intensity. In Fig. 7 experimental data are presented for $\Delta F_{LP}(t)$ measured at different actinic light intensities. The numerical evaluation of these data were performed within the framework of the simplest reaction pattern summarized in Fig. 8. In this scheme k_i represents the rate constants for the different reactions; P-680, Q_A and Z are the well-known redox groups of PS II.

$Na_2S_2O_4$ is assumed to react with Q_A and Z^{ox}, the direct interaction with P-680$^+$ is neglected because of the much faster electron transfer from Z to P-680$^+$.

In order to account for the nonlinear relation between the fluorescence yield and the nonphotochemical quencher ($Pheo^-$) concentration and the existence of α and β centers the same procedure was used as outlined in a previous report for the fluorescence rise from F_0 to F_{max} [20]. The rate constants of the radical pair recombination, $k_b(Pheo)$, the P-680$^+$ reduction by Z, k_z, and the back reaction between Z^{ox} and Q_A^-, $k_b(Q)$, were taken from data reported in the literature [14,16,21–24]. The rate constants of the light reactions k_α and k_β, were derived from fluorescence induction curves ($F_0 \rightarrow F_{max}$) measured in the absence of $Na_2S_2O_4$ at lower actinic light intensities as described in Ref. 13. With k_{Pheo}, $k_{Na_2S_2O_4}$ and $k'_{Na_2S_2O_4}$ as fitting parameters a qualitatively satisfying description of the experimental data could be achieved, as is shown in Fig. 7. The rate constant $k_{Pheo} = 0.01\ s^{-1}$ derived from this analysis is of the same order of magnitude as the rate constant of the fast component of $Pheo^-$ reoxidation after illumination but exceeds that of the slow

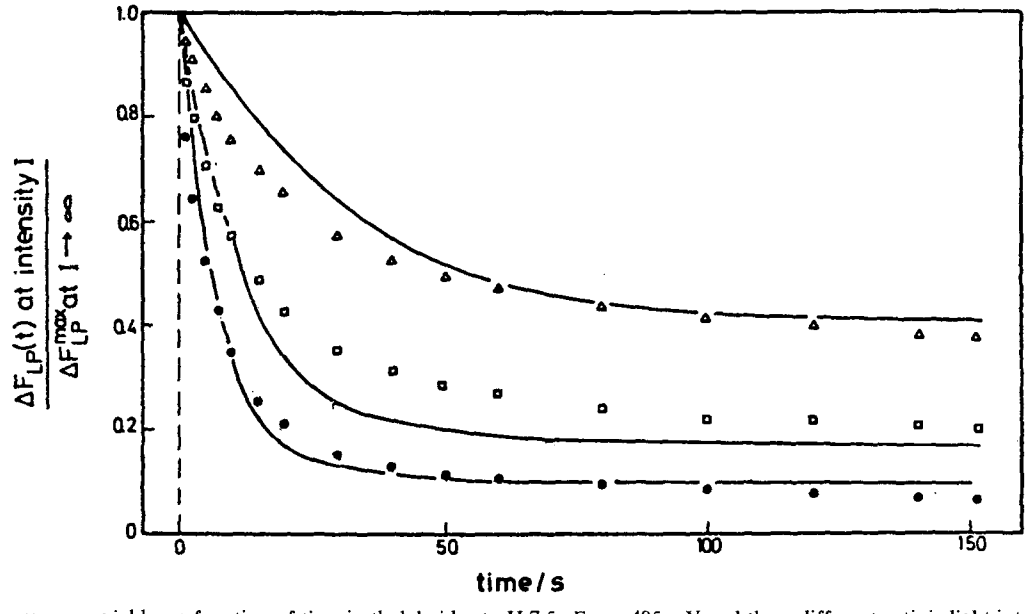

Fig. 7. Fluorescence yield as a function of time in thylakoids at pH 7.5, $E = -405$ mV and three different actinic light intensities. For the sake of comparability the data from the experimental curves are given as symbols: ●, $I = 43\ W/m^2$; □, $I = 23\ W/m^2$ and Δ, $I = 7\ W/m^2$. Rate constants used for the calculations: Light reaction: ●, $k_{app,\alpha} = 440\ s^{-1}$, $k_{app,\beta} = 108\ s^{-1}$; □, $k_{app,\alpha} = 236\ s^{-1}$, $k_{app,\beta} = 58\ s^{-1}$; Δ, $k_{app,\alpha} = 72\ s^{-1}$, $k_{app,\beta} = 18\ s^{-1}$. Dark reactions: $k_{b(Pheo)} = 5.3 \cdot 10^8\ s^{-1}$; $k_Z = 2.8 \cdot 10^5\ s^{-1}$; $k_{-Z} = 30\ s^{-1}$; $k_b(Q) = 6\ s^{-1}$; $k_{Na_2S_2O_4} = 700\ s^{-1}$; $k'_{Na_2S_2O_4} = 700\ s^{-1}$; $k_{Pheo} = 10^{-2}\ s^{-1}$.

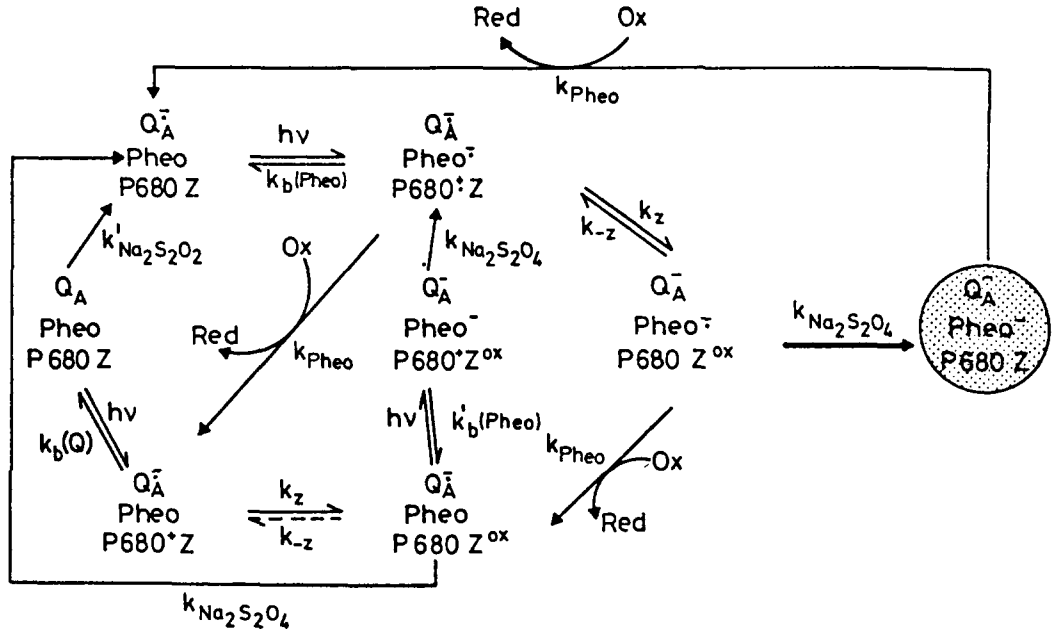

Fig. 8. Simplified scheme of the reaction pattenr in the PS II reaction center complex. The state P-680Pheo$^-$ Q_A^- of nonphotochemical quenching is symbolized by a shaded circle.

component by one order of magnitude. An attempt to use the rate constants of Pheo$^-$ reoxidation calculated from the data of Fig. 6 led to a less close fit (data not shown). The above-mentioned analysis shows that the decline of the fluorescence yield at different actinic light intensities and a constant redox potential can be explained by a slow Pheno$^-$ reoxidation. The same fitting procedure was also used in order to analyze the redox potential dependence of $\Delta F_{LP}^{max}/F_{max}$ at constant actinic light intensity. It was found that this effect can be qualitatively described solely by a redox potential-dependent rate constant k_{Pheo}, whereas the other rate constants were kept the same as in Fig. 7.

Discussion

The present study shows that strong actinic light causes a decline of the fluorescence quantum yield from its maximum level in thylakoids incubated under anaerobic conditions at redox potentials below +150 mV. In the presence of redox mediators two different types of fluorescence decline are observed referred to as $\Delta F_{SQ}(t)$ and $\Delta F_{LP}(t)$, respectively. Both effects are independent of membrane energization by either $\Delta\psi$ or ΔpH and exhibit marked redox potential dependencies. Therefore, they are assumed to be related to light-induced redox reactions: $\Delta F_{SQ}(t)$ probably reflects the interaction with specific quinone derivatives, whereas $\Delta F_{LP}(t)$ is due to photoaccumulation of the redox state P-680Pheo$^-$ Q_A^-.

The redox potential dependence of the maximum extent of $\Delta F_{LP}/F_{max}$ at constant actinic light intensity closely resembles that of the low potential wave of the titration curve for the EPR signal reflecting P-680Pheo$^-$ Q_A^- formation by illumination at 200 K [7]. Therefore, the question arises about the origin of the thermodynamic constraint of Pheo$^-$ photoaccumulation. The analysis of this study shows that a redox-dependent overall rate constant k_{Pheo} qualitatively accounts for the sharp decrease of $\Delta F_{LP}^{max}/F_{max}$ around −300 mV. Two basically different alternatives (mechanisms A and B) could be responsible for this effect (Scheme I).

According to mechanism A, Pheo$^-$ becomes directly oxidized by an exogenous oxidant Ox. In

$$\text{P-680}\ \text{Pheo}^{-}\ \overset{Q_A^{-}}{\xrightarrow{k_{\text{Pheo}}(\text{Ox})}}\ \text{Ox}$$

(mechanism A)

$$\text{P-680}\ \text{Pheo}^{-}\ X_{\text{int}}^{\text{Ox}}\ \overset{Q_A^{-}}{\xrightarrow{k_{\text{Pheo}}(X_{\text{int}})}}\ \text{P-680}\ \text{Pheo}\ X_{\text{int}}^{\text{Red}}\ \overset{Q_A^{-}}{\xrightarrow{k_{X_{\text{int}}}(\text{Ox})}}\ \text{Ox}$$

(mechanism B)

Scheme I

this case the redox potential dependence of the rate constant $k_{\text{Pheo}}(\text{Ox})$ should reflect the exothermicity of the reaction between Pheo^{-} ($E_{m,7}$ around -600 mV, see Refs. 27, 28) and the exogenous component Ox as well as the vibronic coupling properties of the protein environment of Pheo^{-} [25,26]. On the other hand, mechanism B prevails if the protein matrix enwrapping Pheo^{-} forms a tight shield acting as a huge barrier to electron tunneling so that a further intrinsic redox component, X_{int}, is required as mediator for a sufficiently fast Pheo^{-} reoxidation. In this case, the overall rate constant k_{Pheo} reflects the redox potential of the couple $X_{\text{int}}^{\text{ox}}/X_{\text{int}}^{\text{Red}}$. The possibility of an apoprotein acting as a very efficient shield to a redox equilibration with exogenous substances is a well-known phenomenon in PS II for components of the acceptor side (Q_A, see, e.g., Ref. 29) and the donor side (the oxidized form of donor D giving rise to EPR signal II_s [30] is rather stable in the dark, despite its estimated redox potential of $+760$ mV [31].

The data of the present study do not permit an unambiguous distinction between both mechanisms. However, the analysis reveals that the measured titration curve is not necessarily indicative for the existence of an additional intrinsic redox component X_{Int} in the PS II reaction center complex. The implication of this consideration should be taken into account for the interpretation of redox titration curves.

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