Studies on the mechanism of the fluorescence decline induced by strong actinic light in PS II particles under different redox conditions

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Fluorescence changes induced by strong actinic light at room temperature were measured in isolated photosystem II (PS II) particles under different redox conditions. Comparative measurements of the absorption changes accompanying the light-induced fluorescence decay show that in the absence of $Na_2S_2O_4$ the formation of a chlorophyll cation radical (probably together with oxidized carotenoid) causes a quenching while in the presence of $Na_2S_2O_4$ photoaccumulation of the pheophytin anion radical ($Pheo^{\cdot -}$) takes place. The 695 nm band of the 77 K emission spectrum becomes specifically reduced if $Pheo^{\cdot -}$ accumulates in the reaction center, whereas in the case of quenching by the cation radicals the ratio of the emission peaks at 685 nm and 695 nm remains constant. The present data favor the hypothesis that F-695 originates from the PS II reaction center [FEBS Lett. (1982) 147, 16–20]. If the primary plastoquinone acceptor (PQ_A) stayed oxidized in the dark before the onset of the illumination, $Pheo^{\cdot -}$ photoaccumulation was not observed. This effect is explained by the existence of at least one further redox component which is able to accept electrons efficiently from $Pheo^{\cdot -}$. The proposed model also explains the differences in the redox titration curves of the electrochromic bandshift and the initial fluorescence, respectively.

Photosystem II *Fluorescence quenching* *Pheophytin anion photoaccumulation* *77 K emission* *PS II particle*

1. INTRODUCTION

Since the pioneering work of Duysens and Sweers [1], the light-induced fluorescence inductions in algae and chloroplasts were widely used to monitor indirectly the functional state of the PS II reaction center (review [2]). The primary plastoquinone acceptor, PQ_A, was found to be a powerful fluorescence quencher in its oxidized state, while its reduced semiquinone form appears to be inefficient. Accordingly, during actinic illumination, the fluorescence rises from the initial level, F_0, to its maximum, F_{max}, if PQ_A becomes photochemically reduced. The quenching efficiency of the transiently formed Chl a_{II}^{+} radical of the reaction center does not affect the commonly measured fluorescence induction curves because of the very fast turnover kinetics of Chl a_{II} [3]. If PQ_A is chemically reduced by dithionite, strong actinic illumination of oxygen-evolving PS II particles was found to cause a fluorescence decline almost down to the level of F_0 [4]. In contrast to the very efficient fluorescence rise due to photochemical PQ_A reduction, the quantum yield of this fluorescence decrease appears to be rather low ($\leq 10^{-2}$ [4]). These findings led to a reinterpretation of the fluorescence induction, based on the assumption that pheophytin, Pheo, acts as intermediary redox component for the photoinduced electron transfer from the Chl a_{II}^{*} excited singlet state to PQ_A. It has been postulated that F_{max} is ac-

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tually a very fast delayed fluorescence (rather than prompt fluorescence) due to the recombination between Chl a_{II}^{+} and Pheo$^{\cdot -}$, with τ = 2–4 ns, giving rise to efficient singlet exciton formation so that the lifetime closely resembles that of prompt fluorescence. The light-induced fluorescence decline under redox conditions of PQ_A being chemically reduced was referred to be due to the low quantum yield accumulation of the state Chl $a_{II}\cdot$Pheo$^{\cdot -}\cdot PQ_A^{\cdot -}$ with Pheo$^{\cdot -}$ acting as efficient fluorescence quencher. Recently, the hypothesis was proposed that the singlet exciton formation by the back-reaction between Chl a_{II}^{+} and Pheo$^{\cdot -}$ is created at the pheophytin, which gives rise at 77 K to the fluorescence emission at 695 nm [5].

The present paper analyzes the fluorescence changes induced by strong actinic light under different conditions in isolated PS II particles.

2. MATERIALS AND METHODS

PS II particles were prepared either from local market spinach as in [6] or from a thermophilic cyanobacterium *Synechococcus vulcanus* Copeland by a procedure similar to [7] as in [8]. The standard reaction mixture for the fluorescence measurement contained: PS II particles (5 μg Chl/ml), 2 mM $MgCl_2$, 10 mM NaCl, 0.4 M sucrose and 50 mM Hepes (pH 7.8). For the measurements of light-induced difference spectra, chlorophyll was at 13 μg/ml and in order to prevent particle aggregation $MgCl_2$ was omitted. Fluorescence transients at room temperature were measured by a conventional technique as in [9]. Strong actinic broad-band blue light (100 mW/cm^2) was applied; 77 K fluorescence emission spectra were measured by a Shimadzu spectrofluorometer (model 502) with the same samples which were used before for the detection of the fluorescence transients. The samples were transferred to 400-μl cuvettes (thickness 1 mm) and rapidly frozen. Fluorescence, excited by 435 nm light (bandwidth 10 nm) and emitted from the surface, was detected after passing the monochromator ($\Delta\lambda$ = 3 nm) by a multiplier. The difference spectra were recorded by a commercially available Shimadzu spectrophotometer, model UV-3000 and by illuminating the probe cuvette in the sample holder with light from a projector lamp before the measurement, while the reference cuvette was kept in darkness.

3. RESULTS

The first problem to be clarified was whether Pheo$^{\cdot -}$ can be accumulated in the reaction center, if the primary plastoquinone acceptor, PQ_A, is not chemically pre-reduced but becomes photochemically reduced. Experiments performed with normal chloroplasts under either aerobic conditions or N_2 did not reveal a significant fluorescence decrease (data not shown) which can be ascribed to Pheo$^{\cdot -}$ quenching. In order to prevent $PQ_A^{\cdot -}$ reoxidation by the back reaction with the redox state S_2 of the water-oxidizing enzyme system Y, the most powerful ADRY agent [10] 2-(3-chloro-4-trifluoromethyl)-anilino-3,5-dinitrothiophene (ANT 2p) was applied, which fixes system II in the state $S_1PQ_A^{\cdot -}$ if DCMU is present [9]. No stimulation of the fluorescence decrease was observed under these conditions (data not shown). This indicates that Pheo$^{\cdot -}$ cannot be photoaccumulated in the reaction center, unless PQ_A is chemically pre-reduced in the dark. These findings correspond with data gathered from chloroplasts illuminated at lower temperatures [11]. Latest EPR measurements also reveal that light-induced Pheo$^{\cdot -}$ formation cannot be achieved after PQ_A photoreduction (A.W. Rutherford, in preparation).

A simpler situation was anticipated to arise in spinach PS II preparations with high oxygen-evolving capacity, because in the absence of exogenous acceptors, the electron efflux from $PQ_A^{\cdot -}$ is very sluggish due to the rather slow PQ-pool oxidation (unpublished data). Typical traces of fluorescence transients, illuminated by bright actinic light (100 mW/cm^2), are depicted in fig.1.

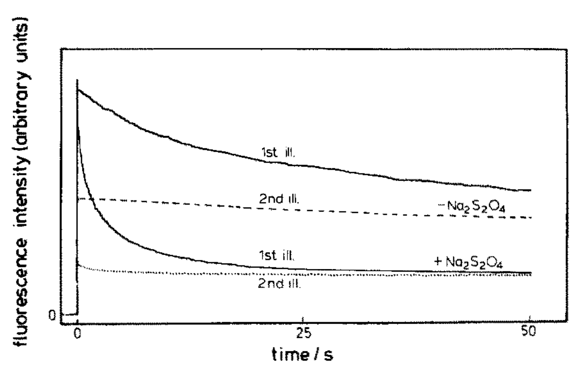

Fig.1. Fluorescence yield as function of illumination time with strong blue actinic light in spinach PS II particles.

The data show that, even in the absence of $Na_2S_2O_4$, a remarkable fluorescence decline is observed (the fast rise due to the reduction of the acceptor side is not resolved in this time scale), which appears to be almost irreversible as shown by a second illumination (– – –). In the presence of 4 mg/ml $Na_2S_2O_4$, a much steeper fluorescence decline is observed. Surprisingly, the effect is often nearly irreversible (fig.1, $\cdots$). This high extent of irreversibility, however, was rather specific for the PS II particles obtained from spinach. Analogous experiments performed with PS II particles isolated from a thermophilic blue-green cyanobacterium (*Synechococcus*) revealed a remarkable degree of reversibility (fig.2). Furthermore, both preparations differ in the kinetics of the fluorescence decline. The kinetics are almost monoexponential in the *Synechococcus* PS II particles, while a more complex behavior is observed in the spinach preparations (data not shown). Latest EPR data indicate, that in PS II particles from spinach, illumination in the presence of $Na_2S_2O_4$ leads to further reduction of $PQ_A^{\cdot -}$ into its quinol form which remains rather stable unless exogenous oxidants are added [12]. This effect might explain the 'irreversibility' of $Pheo^{\cdot -}$ photoaccumulation as well as the differences for the various preparations.

The fluorescence decline observed in spinach PS II particles in the absence of $Na_2S_2O_4$ is slightly

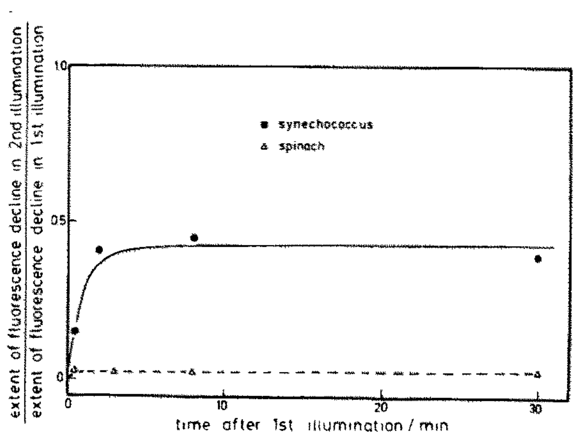

Fig.2. Extent of fluorescence decline in the second illumination related to that of the first illumination as a function of time between first and second illumination of PS II particles in the presence of 4 mg/ml $Na_2S_2O_4$. Experimental conditions as in fig.1.

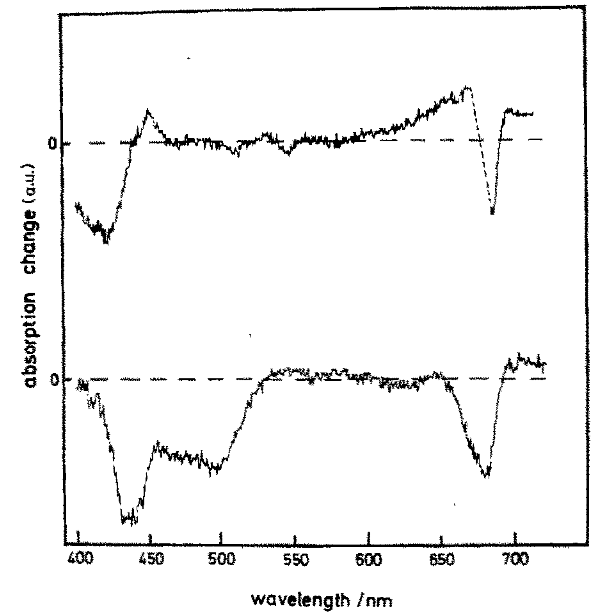

Fig.3. Difference spectra of spinach PS II particles illuminated with strong actinic white light vs dark samples. The probe cuvette was illuminated for 2 min with light from a projector lamp passed through a heat filter. Other conditions as described in section 2: top, in the presence of 4 mg/ml $Na_2S_2O_4$; bottom, without $Na_2S_2O_4$.

enhanced by 1 mM $K_3Fe(CN)_6$. Accordingly, it appears rather unlikely that this phenomenon is caused by a quenching due to $Pheo^{\cdot -}$ accumulation during the illumination. The irreversibility of the effects either in the presence or absence of $Na_2S_2O_4$ offers a possibility for monitoring directly the bright actinic light-induced difference spectra which accompany the fluorescence declines depicted in fig.1. The results obtained are shown in fig.3. The spectrum at the top, observed in the presence of 4 mg/ml $Na_2S_2O_4$, reveals the typical difference spectrum for pheophytin anion formation [4] and completely agrees with previous findings of $Pheo^{\cdot -}$ accumulation. It roughly corresponds to 1 $Pheo^{\cdot -}$/400 Chl (a small fluorescence artefact arising around 685 nm is indicated by the dashed line). A completely different spectrum, however, is obtained in the absence of $Na_2S_2O_4$ (fig.3, bottom). This spectrum can be interpreted as the bleaching of a Chl *a*, probably accompanied by a carotenoid bleaching (manifested by the bleaching in the range 450–500 nm). As this reaction also occurs under rather oxidizing conditions, it is assumed to indicate the formation of cation radicals (1–2 species/reaction center) which are responsible for the fluorescence decline. Measurements of the fluorescence induction curves of the same samples under much lower actinic light intensity ($\leqslant 1\ mW/cm^2$) indicate that the photooxidatively induced fluorescence decline (fig.1) mainly affects the maximum fluorescence, F_{max}, while the initial fluorescence, F_0, becomes much less reduced (data not shown). The small effect on F_0 excludes an unspecific bulk pigment bleaching, similar to that found in Tris-treated chloroplasts [13] as being mainly responsible for the observed fluorescence decline. As the electron transport activity from H_2O to DCIP is impaired to a markedly smaller extent (about 25% inhibition) than the variable fluorescence ($F_v = F_{max} - F_0$), it appears reasonable to assume that light-induced cation radical formation primarily stimulates nonradiative exciton dissipation. The idea of a photooxidative quencher generation is supported by the finding that subsequent addition of $Na_2S_2O_4$ leads to 70–80% recovery of the original maximum fluorescence level (data not shown). The species giving rise to the radicals are not yet identified, but the difference spectrum of fig.3 favors a special Chl *a* and a carotenoid as likely candidates. Both types of species were found to be photooxidizable by PS II under different conditions [14,15]. Regardless of these details, the fluorescence decreases caused by bright light in the absence and presence of $Na_2S_2O_4$ (fig.1) appear to be completely different with respect to the underlying mechanism. Accordingly, if one takes into consideration a recent hypothesis about the origin of the 695 nm fluorescence emission [5] the question arises whether the 77 K emission spectra differ from samples illuminated by strong light in the presence and absence of $Na_2S_2O_4$, respectively. The results are depicted in fig.4. The control samples, frozen under very dim light to 77 K, give rise to spectra which are unchanged by $Na_2S_2O_4$ addition (in fig.4b, for the sake of direct comparison with the illuminated sample (4c) only the signal in the presence of $Na_2S_2O_4$ is shown). The emission spectrum of spinach PS II preparations, illuminated in the absence of $Na_2S_2O_4$, remains practically unaffected in its shape, while the intensity is reduced due to the diminished fluorescence

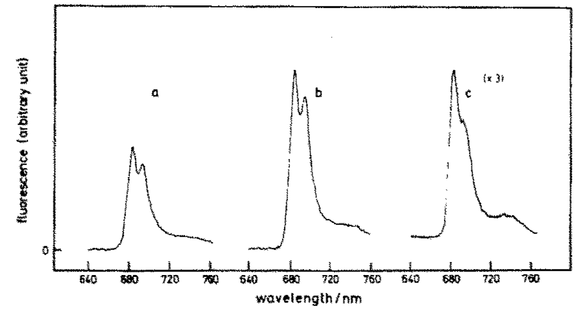

Fig.4. Fluorescence emission spectra of spinach PS II particles frozen at 77 K: (a) sample without $Na_2S_2O_4$, illuminated at room temperature for 2 min with strong blue light ($100\ mW/cm^2$) before freezing to 77 K; (b) sample in the presence of 4 mg/ml $Na_2S_2O_4$, without actinic light preillumination; (c) sample in the presence of 4 mg/ml $Na_2S_2O_4$, illuminated at room temperature for 2 min with strong actinic blue light ($100\ mW/cm^2$) before freezing to 77 K (3-fold sensitivity for recording). Other experimental conditions as section 2.

quantum yield (see fig.1). A markedly different fluorescence spectrum, however, is observed in samples illuminated in the presence of 4 mg/ml $Na_2S_2O_4$. For the sake of a direct comparison, the spectrum in fig.4c is enlarged 3-times in its intensity scale. It shows a drastic reduction of the 695 nm emission band compared to that peaking around 685 nm. A specific reduction of the 695 nm band is not only observed after illumination at room temperature in the presence of $Na_2S_2O_4$ but can also be achieved directly by illumination at 77 K (D. Kyle and J. Bretons group, personal communication).

4. DISCUSSION

These results indicate that the PS II reaction centers cannot be trapped photochemically at room temperature in the state Chl $a_{II} \cdot Pheo^{\cdot -} \cdot PQ_A^{\cdot -}$ under redox conditions where PQ_A is oxidized in the dark before the onset of bright light illumination, even in the presence of DCMU and ANT 2p which highly stabilize photoreduced $PQ_A^{\cdot -}$ [9]. This conclusion is in line with latest EPR measurements of Rutherford (in preparation). On the other hand, the almost irreversible photoaccumulation of $Pheo^{\cdot -}$ in the presence of $Na_2S_2O_4$ suggests that pheophytin is buried deeply within the protein matrix of the reaction center, preventing a direct redox equilibration with the ambient suspension. Both findings together lead to the conclusions that:

(1) There exists at least one further endogenous redox component which can efficiently accept electrons from photoreduced $Pheo^{\cdot -}$, even if $PQ_A^{\cdot -}$ stays reduced;

(2) $Pheo^{\cdot -}$ can equilibrate with the exogenous medium only through these endogenous components.

A likely candidate could be the acceptor component discovered recently [16,17]. Accordingly, $Pheo^{\cdot -}$ photoaccumulation can only occur if this component is reduced prior to the illumination. If, furthermore, this component is assumed to act also as fluorescence quencher in its oxidized state, the double-wave redox titration curve of the initial fluorescence could be understood to reflect the chemical reduction of PQ_A and the above-mentioned redox component. This interpretation also readily explains the single-wave redox behavior of the electrochromic bandshift [18], because only the electron transfer to PQ_A was found to cause a 'stable' transmembrane electric potential gradient [16,17]. Based on the above-mentioned idea, the redox potential of the postulated redox component is estimated to be of the order of -300 mV. This assumption is in line with the latest findings that 70–80% of the total $Pheo^{\cdot -}$ photoaccumulation at 200 K is dependent on the chemical reduction of a redox component with $E_{m,7} \approx -275$ mV [11]. Therefore, it is concluded that the majority of the PS II reaction centers contain this component, while the remaining 20–30% do not, so that $Pheo^{\cdot -}$ accumulation in this case requires only the prereduction of PQ_A [11] with a redox potential around 0 V [18]. The results here also show that $Pheo^{\cdot -}$ photoaccumulation does not only drastically reduce the fluorescence quantum yield, but in addition significantly changes the 77 K emission spectrum; i.e.,the 695 nm band is seriously diminished. This finding favors the idea that at least a significant part of the 695 nm emission has its origin in the reaction center and that $Pheo^{\cdot -}$ specifically suppresses this emission, in line with Breton's hypothesis. However, further experiments are required to clarify this point unambiguously.

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