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PICOSECOND FLUORESCENCE KINETICS IN SPINACH CHLOROPLASTS AT ROOM TEMPERATURE

EFFECTS OF PHOSPHORYLATION

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We have used single-photon timing with picosecond resolution to investigate the effect of phosphorylation on the fluorescence decay from broken spinach chloroplasts. Phosphorylation of spinach thylakoids causes a quenching of the slow decay phase (equivalent to a quenching of variable fluorescence) and an increase in the yield of the middle phase decay component. In addition, phosphorylation alters the intensity dependence of fluorescence in a manner which indicates a decreased antenna size of Photosystem II. The observed changes are indicative of a State 1–State 2 transition and show a clear reversal when the membranes are dephosphorylated.

Introduction

The room-temperature fluorescence emission from higher plant tissues reflects the state of and interaction between the photochemical reaction centers of PS II [1]. In the photoactive (open) condition the fluorescence yield is low, the so-called F_o level; when the reaction centers are closed to photochemistry the fluorescence yield is large (F_{max}). These changes in fluorescence yield have been related to the redox state of the electron acceptor Q [2]. As this acceptor becomes reduced by photochemistry the fluorescence yield increases by a factor of 3–5 [3,4]. In addition to the redox state of Q, the room-temperature fluorescence emission is sensitive to the transfer of excitation energy between PS II reaction centers [1]. Joliot and Joliot [1] made this deduction from the observation that fluorescence induction curves are sigmoidal rather than exponential. Further analysis indicated that the induction curve is biphasic and consists of a fast (α) component and a slow (β) component [5,6]. The α-component arises from a connected 'matrix' of PS II centers, while the β-component originates in separate PS II units [5–7]. Because these factors influence fluorescence, analysis of the decay kinetics of this emission should reflect both the photochemical activity and the transfer of excitation energy within the pigment bed [8,9].

Haehnel et al. [8] used single-photon counting with picosecond resolution to investigate the decay kinetics of fluorescence emission from a variety of photosynthetic organisms. From this work it was

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Abbreviations: PS, photosystem; Chl, chlorophyll; Tricine, *N*-tris(hydroxymethyl)methylglycine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; LHC, light-harvesting chlorophyll-protein complex.

proposed that the fluorescence decay is best characterized by three exponential components [8,9]. The slowest phase (1–2 ns) is due to the radical-pair recombination of P-680$^+$ and I$^-$ in the PS II center. This recombination occurs with a high yield when the acceptor Q is reduced and is comparable with the variable component of room-temperature fluorescence. The two faster phases are due to excitation that is lost prior to reaching the reaction center. The fastest phase (50–100 ps) is kinetically controlled by the rate of excitation transfer from the Chl *a* antenna of PS II (Chl a_2) to the reaction center of PS II. The middle phase (300–750 ps) is kinetically controlled by the rate of excitation transfer from the Chl *a/b* light-harvesting antenna to the reaction center of PS II. Nairn et al. [9] described effects of Mg^{2+} concentration on these decay components. The changes observed in that study have been linked to the phenomena of state changes, first described by Murata [10] and Bonaventura and Myers [11]. There is now a wide body of evidence to suggest that state transitions in vivo are not primarily caused by variations in the stromal concentration of cations, but by the light-induced phosphorylation of the protein of the light-harvesting Chl *a/b* complex, LHC II [12–17]. These reversible phosphorylation-induced state transitions have been observed to affect the level of F_{max} [18,19] and the amount of α- and β-components that constitute the F_{max} yield [19]. These changes have been interpreted as indicating increased energy transfer in favor of PS II [15,19]. In addition to a quenching of the F_{max} yield there is evidence that phosphorylation leads to a decrease in the F_0 level [14], although this effect also appears to be sensitive to cation concentration and may be more complex in origin [20].

In this paper we describe the reversible effects of protein phosphorylation on the fluorescence decay kinetics of spinach thylakoids. We explain these observations in terms of State 1–State 2 transitions and postulate a mechanism by which they might occur.

Materials and Methods

Fresh-grown spinach was harvested, and chloroplasts were isolated [19] in 15 mM Tricine-NaOH (pH 7.8), 0.4 M sorbitol and 10 mM NaCl. The pellet was washed in hypotonic buffer: 10 mM Tricine-NaOH (pH 7.8), 10 mM NaCl and 5 mM $MgCl_2$ to rupture the envelope membrane and was then resuspended in 10 mM Tricine-NaOH (pH 7.8), 0.1 M sorbitol, 10 mM NaCl and 5 mM $MgCl_2$. Four aliquots of this resuspension mixture were diluted to 100 μg/ml Chl and were treated as follows [19,21]. (i) The dark + ATP control was treated with ATP (Sigma Chemical Co.) (final concentration 100 μM) and incubated at room temperature for 20 min in the dark. NaF was then added (final concentration 10 mM) to inhibit phosphatase enzymes [21], and the incubation was continued for a further 30 min. (ii) Light – ATP control samples were prepared by incubation in cool white light [21] for 20 min in the absence of ATP; 10 mM NaF was then added and the sample incubated in the dark for a further 30 min. (iii) Phosphorylated membrane samples were prepared by incubation in the light plus 100 μM ATP for 20 min then treated with NaF as in treatments i and ii. (iv) Dephosphorylated membranes were prepared as were phosphorylated membranes except that addition of 10 mM NaF was made after the 30 min incubation in the dark to allow dephosphorylation [21]. All samples were then pelleted by centrifugation for 10 min at 10000 × *g*, and washed in buffer: 10 mM Tricine-NaOH, 0.1 M sorbitol, 5 mM $MgCl_2$ and 10 mM NaF. Samples were resuspended in the same buffer to a concentration of 200 μg/ml Chl prior to dilution to 15 μg/ml Chl for fluorescence measurement. The fluorescence measurements were made in the absence of DCMU, as described in Ref. 22. Chlorophyll determinations were made by the procedure of Mackinney [23].

Picosecond fluorescence decay kinetics were measured as described elsewhere [8,9,22] and resolved using a three-exponential deconvolution [8]. Fluorescence was excited through a 620 nm narrow band-pass filter, using neutral density filters to vary the intensity of the exciting beam and/or fluorescence emission. Emission was measured at 680 nm. For F_0 measurements neutral density filters were placed in the excitation beam to give approx. 1% maximum laser intensity. Fluorescence at the F_{max} level was measured with 100% laser intensity. Fluorescence yields were calculated from the nor-

TABLE I

LIFETIMES AND YIELDS OF THE F_0 LEVEL FLUORESCENCE FROM SPINACH THYLAKOIDS

Samples contain 15 μg/ml Chl in 10 mM Tricine-NaOH (pH 7.8), 0.1 M sorbitol, 5 mM $MgCl_2$ and 10 mM NaF.

Treatment	τ_f (ps)	ϕ_f	τ_m (ps)	ϕ_m	τ_s (ps)	ϕ_s	ϕ_t
Light − ATP	75	1.8	390	12.8	1140	4.7	19.3
Dark + ATP	75	1.7	380	12.6	1200	3.5	17.8
Phosphorylated	90	2.4	380	12.0	1400	3.1	17.5
Dephosphorylated	80	1.7	380	11.7	1200	4.0	17.4

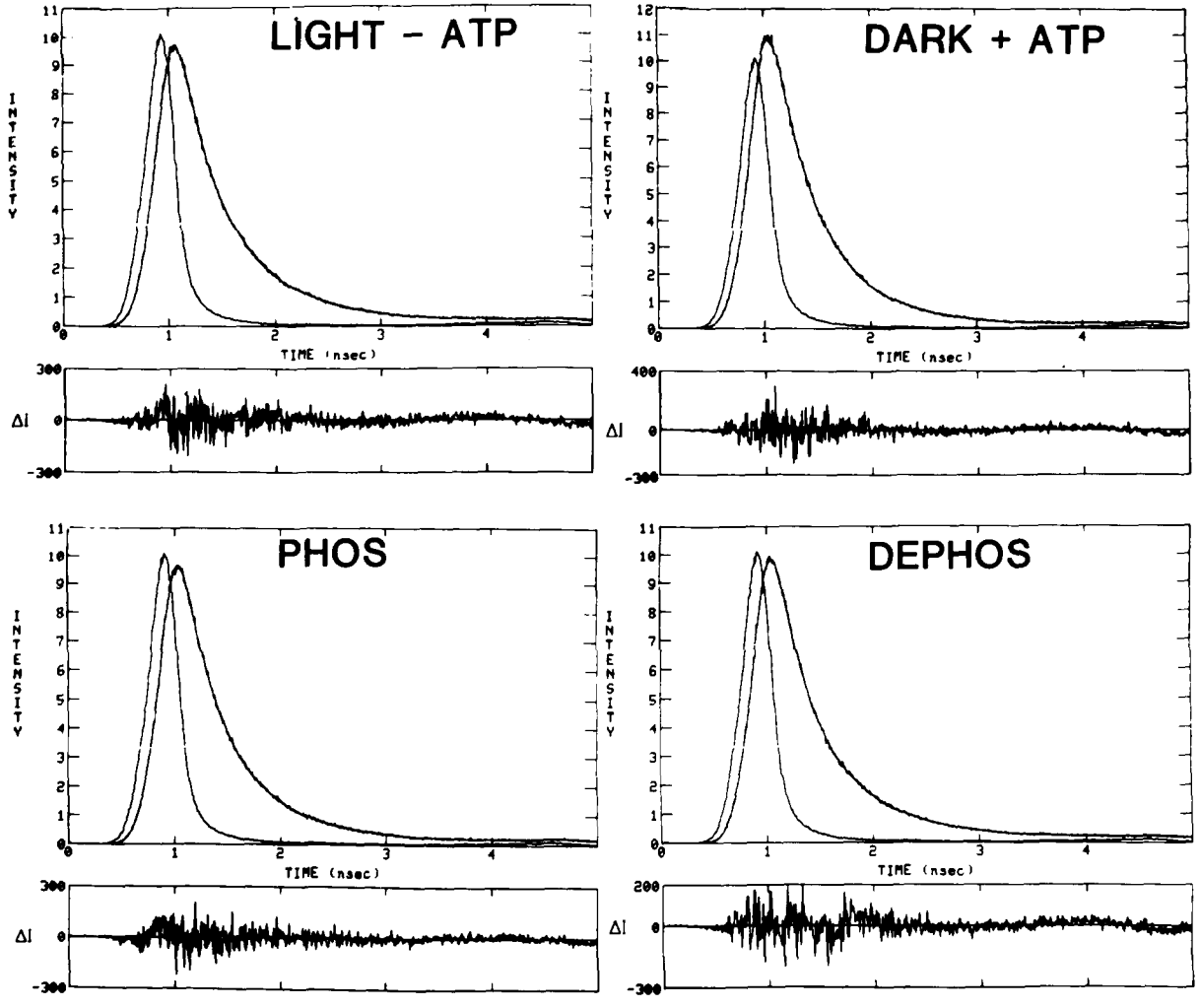

Fig. 1. Fluorescence decay of spinach thylakoids observed at 680 nm, excitation 620 nm, under the F_0 condition. Experimental fluorescence decays are superimposed with a smooth curve calculated from the best fit of a three-exponential decay. The differences between the decay and the best three-exponential fits (ΔI) are shown below. Experimental conditions defined in Materials and Methods. (DE) PHOS, (de)phosphorylated.

malized fluorescence yields [8] by correction for the transmission of the various filters used.

Results

Effect of phosphorylation on F_0 level fluorescence

The results of the three-exponential deconvolution of fluorescence decay at the F_0 level under the four experimental conditions are summarized in Table I. Examples of the decay deconvolution are shown in Fig. 1. The two experimental control conditions, light − ATP and dark + ATP, have similar composition, with the exception of an increased yield of the slow phase in the light − ATP control. This increase in the slow phase yield is reflected in the total fluorescence yield at F_0 and may relate to the phenomenon of photoinhibition induced by exposure to light at room temperature, as discussed by Horton and Black [20]. Phosphorylation of the thylakoids produces distinct changes in the fluorescence. There is a quenching of ϕ_t and ϕ_s in comparison to the light − ATP control, but little effect compared to the dark + ATP control. The lifetime of the slow component is increased by phosphorylation from 1.1–1.2 to 1.4 ns. Upon dephosphorylation there is a noticeable decline in τ_2, while ϕ_s and ϕ_t increase towards the values observed in the control. The deviations from best fit obtained in the experiments shown in Fig. 1 are compatible with those presented by Haehnel et al. [8], which is suggestive that no new components are introduced by phosphorylation.

Effect of phosphorylation on F_{max} level fluorescence

Upon closing the reaction centers of PS II by exposure to high light intensity, characteristic changes in the composition of the fluorescence decay occur [8,9]. The closing produces some minor changes in the fast and middle components, but there is a dramatic increase in the yield of the slow component, accompanied by an increase in its lifetime (see Fig. 2 and Table II). In particular, we find that the same changes occur in both the control conditions (Table II). As observed at the F_0 level, the total fluorescence yield of the light − ATP condition is greater than that of the dark + ATP control. This difference in ϕ_t again results from an enhanced slow phase yield. The lifetimes of the three components are, within instrument limitations [8], the same in both control conditions. The ratio of F_{max}/F_0 yields in the controls is 3.9 for light − ATP and 3.8 for dark + ATP. These values are indicative of State 1 [9,10].

The F_{max} fluorescence of phosphorylated membranes differs from that in the control conditions in several important ways. In agreement with the data obtained from peas by Kyle et al. [19], there is a 20–30% decrease in the total F_{max} yields. The decline arises from a loss of the slow phase. Unlike the decay composition under control conditions the lifetime of the slow phase (τ_s) is not altered from the F_0 level, and at 1.4 ns is slightly shorter than the τ_s of 1.5–1.6 ns observed in these controls. Another major difference in the phosphorylated membranes is the increased yield of the middle phase (ϕ_m), suggesting a decrease in the efficiency of energy transfer from LHC II to the reaction center of PS II. In phosphorylated membranes ϕ_m comprises 20% of ϕ_t, yet under control conditions ϕ_m is less than 10% of ϕ_t. In contrast to the observations of Horton and Black [20], phosphorylation decreases the F_{max}/F_0 ratio to 3.0 in this study, which is indicative of the State 2 condition [9,19]. This phosphorylation-induced quenching of variable fluorescence F_v (where $F_v = F_{max} - F_0$, and is equivalent to the slow phase yield) was

TABLE II

LIFETIMES AND YIELDS OF THE F_{max} LEVEL FLUORESCENCE FROM SPINACH THYLAKOIDS

Samples contain 15 μg/ml Chl in 10 mM Tricine-NaOH (pH 7.8), 0.1 M sorbitol, 5 mM $MgCl_2$ and 10 mM NaF.

	τ_f (ps)	ϕ_f	τ_m (ps)	ϕ_m	τ_s (ps)	ϕ_s	ϕ_t
Light − ATP	65	1.0	470	6.3	1580	67.1	74.4
Dark + ATP	60	0.8	470	5.7	1500	61.4	67.9
Phosphorylated	65	1.3	500	10.4	1390	41.0	52.7
Dephosphorylated	60	0.9	480	6.7	1520	54.2	61.8

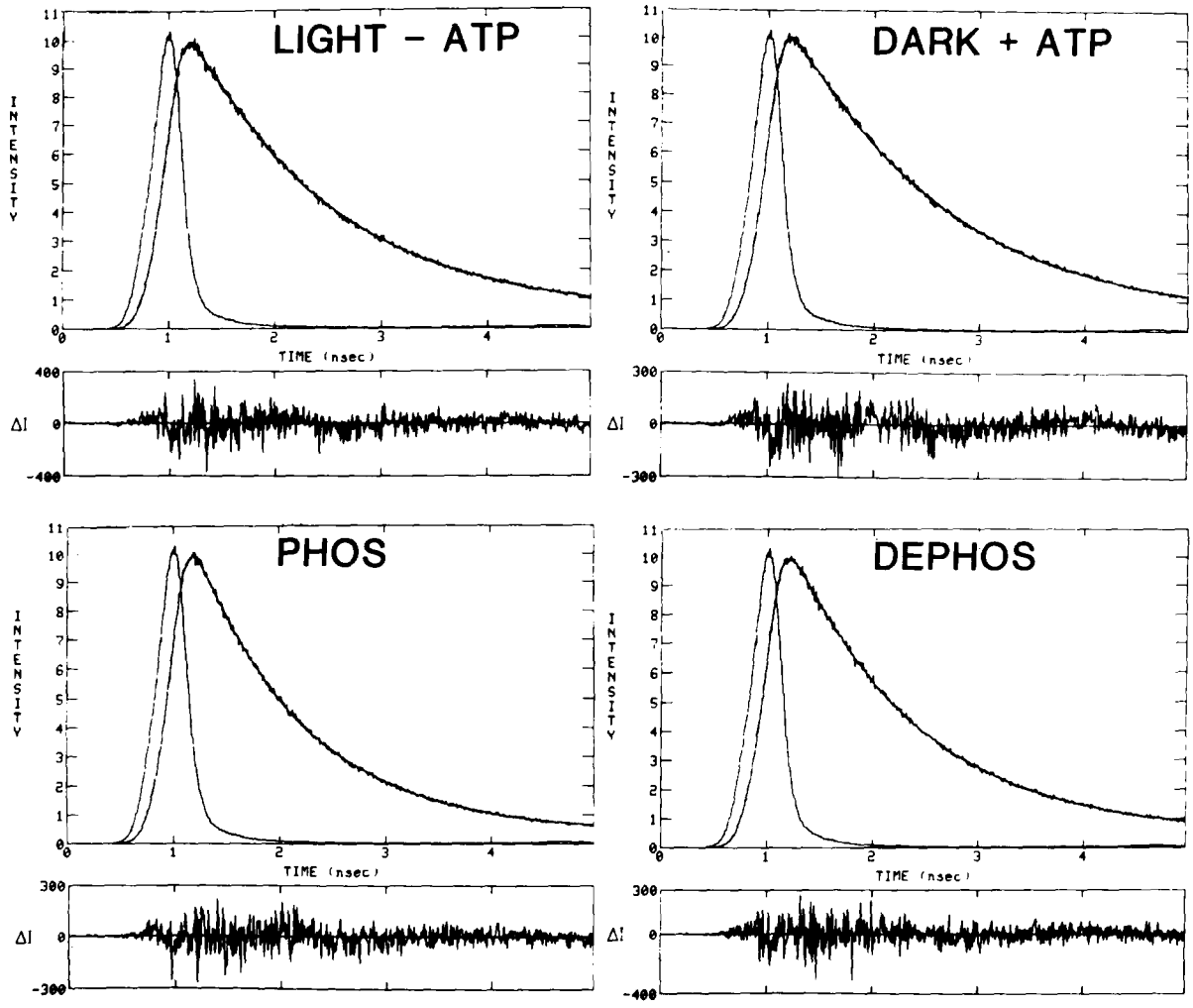

Fig. 2. Fluorescence decay of spinach thylakoids at 680 nm, excitation 620 nm, under the F_{max} condition. (Other details as shown in Fig. 1.)

previously reported by Bennett et al. [18] and Kyle et al. [19]. Dephosphorylation of the membranes causes ϕ_s to recover about 50%, while τ_s is again sensitive to the redox state of Q and increases from 1.2 ns at F_0 to 1.5 ns at F_{max}. The yield of the middle phase is decreased to the levels observed under control conditions. These changes indicate the reversible nature of the phosphorylation effect on room-temperature fluorescence [19], although under these experimental conditions 100% recovery is not observed in spinach thylakoids. The ratio of F_{max}/F_0 emission calculated for dephosphorylated membranes from these data is 3.4.

Effect of phosphorylation on the intensity dependence of fluorescence decay kinetics

The effect of incident light intensity on the fluorescence emission properties of light – ATP-treated membranes is shown in Fig. 3. The lowest light intensity corresponds to the F_0 level, the highest intensity to F_{max}. The total fluorescence yield (ϕ_t) exhibits the expected [8] sigmoidal dependence on light intensity; this increase in ϕ_t is parallel to the increase of ϕ_s. Phosphorylation of the membranes causes the intensity dependence of ϕ_t and ϕ_s to become less sigmoidal (Fig. 4). The yield of the middle phase increases at low intensity

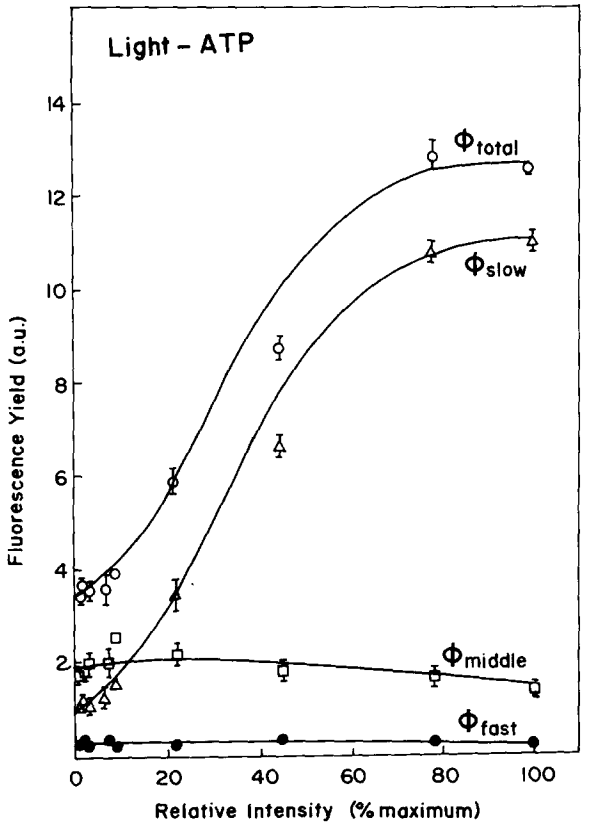

Fig. 3. Yields of components of the fluorescence decay in light – ATP spinach thylakoids as a function of laser intensity. Points are presented as means and standard errors of the mean. (●) Fast phase, (□) middle phase, (△) slow phase, and (○) total fluorescence).

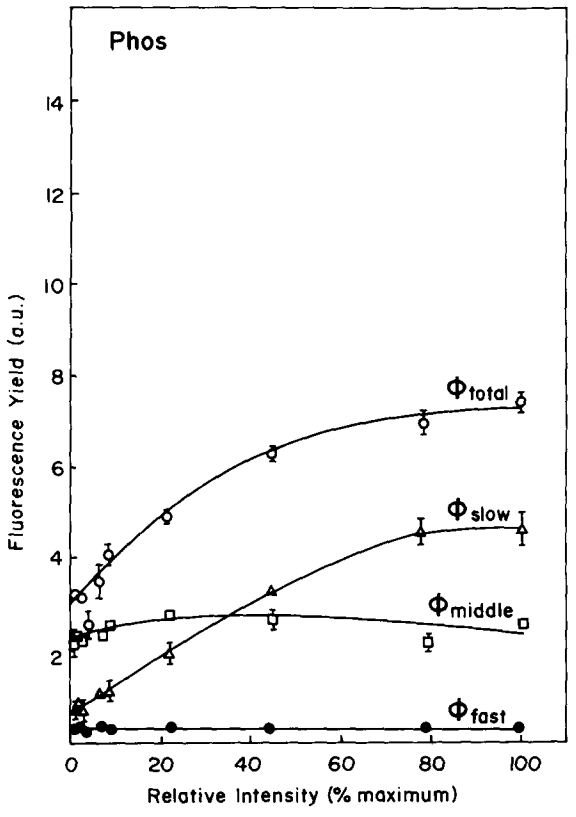

Fig. 4. Yields of components of the fluorescence decay in phosphorylated spinach thylakoids as a function of laser intensity. (For legend, see Fig. 3.)

but does not decline as the intensity is further increased. The intensity dependence of fluorescence from dephosphorylated membranes is shown in Fig. 5, where we observe that both ϕ_t and ϕ_s return to a sigmoidal response to increasing light intensity. The characteristic rise and dip of ϕ_m also return upon dephosphorylation.

These data are clearly in accord with previous observations. Phosphorylation causes a reversible quenching of ϕ_s and an enhanced yield of the middle phase. The quenching of ϕ_t and ϕ_s is indicative of a phosphorylation-induced State 1–State 2 transition, and the increase in ϕ_m may reflect the mechanism by which they are accomplished. The plots of fluorescence yield vs. intensity (Figs. 3–5) show changes not only in the yield of fluorescence but also in the rates at which these levels are achieved. The shift from a sigmoidal to a hyperbolic response must arise from reorganization of the pigment bed, functionally if not structurally, which is indicative of a mechanism for state transitions.

Because variable fluorescence is believed to arise from closed reaction centers [1,2,5,6], a plot of F_v vs. intensity should best demonstrate the different rates of reaction center closing in each condition. Such a plot is shown in Fig. 6 for light – ATP, phosphorylated and dephosphorylated membranes. These data reveal two important facts. The initial rate of rise of F_v is greater for light – ATP and dephosphorylated membranes than for phosphorylated membranes, and the reaction center closing is initiated at higher intensities in phosphorylated membranes than under the other conditions (see inset). This second observation clearly

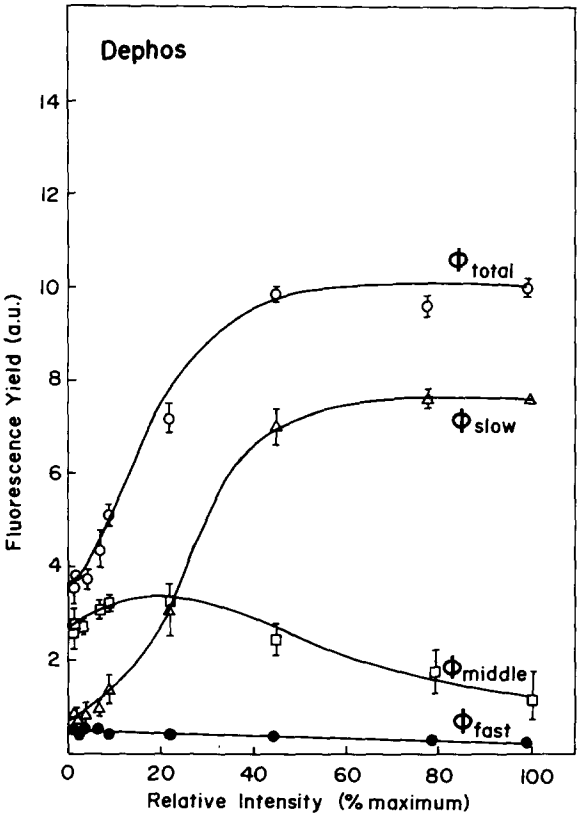

Fig. 5. Yields of components of the fluorescence decay in dephosphorylated spinach thylakoids as a function of laser intensity. (For legend, see Fig. 3.)

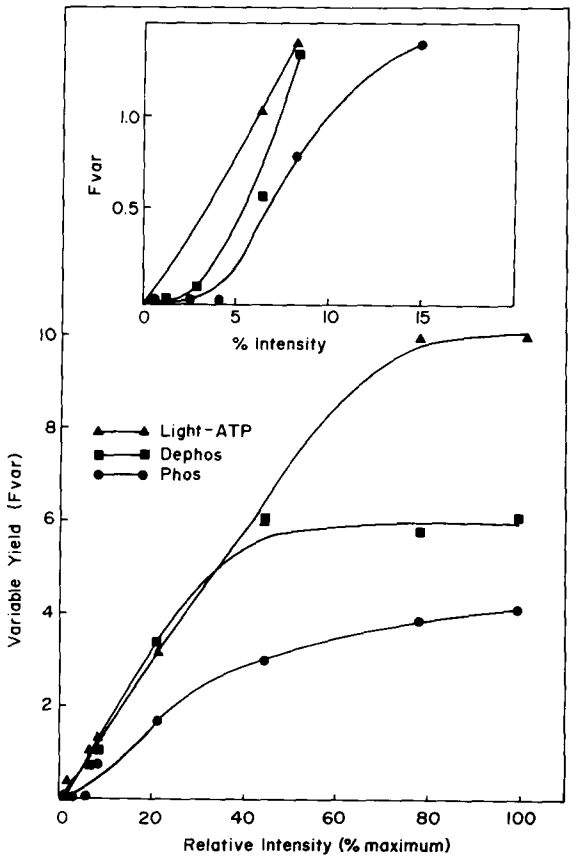

Fig. 6. Plots of variable fluorescence yield ($F_v = F_{max} - F_0$) as a function of laser intensity, for light − ATP, phosphorylated and dephosphorylated membranes. The inset depicts an expanded plot near the origin. (▲———▲) Light − ATP membranes, (■———■) dephosphorylated membranes and (●———●) phosphorylated membranes.

demonstrates a reduced antenna size in the case of phosphorylated membranes. These differences are perhaps clearer in a double-reciprocal plot of the same data (see Fig. 7). Such a plot can be compared to a Lineweaver-Burk plot for enzyme kinetics; the y-axis intercept is a reciprocal measure of the amount of variable fluorescence, which is a measure of PS II activity, while the slope is an inverse measure of antenna size and/or the altered efficiency of excitation transfer. From these data we suggest that the amount of reaction center closing in phosphorylated membranes is only half that of the control or dephosphorylated rate. Similarly, the relative antenna size of light − ATP membranes and dephosphorylated membranes is approximately twice that of the phosphorylated membranes.

One final observation that we report in this paper is a characteristic response of τ_s (slow phase lifetime) to increasing light intensity. In all of the experiments that we performed it was noted that τ_s underwent an initial decline with increasing intensity from the F_0 level. An example of this response is shown in Fig. 8. After the initial decline the lifetime showed a steady rise as the F_{max} level was approached. This observation suggests a possible heterogeneity of the slow phase fluorescence decay; the origin of ϕ_s under the F_0 condition may be different from the chief contribution to ϕ_s in the F_{max} state.

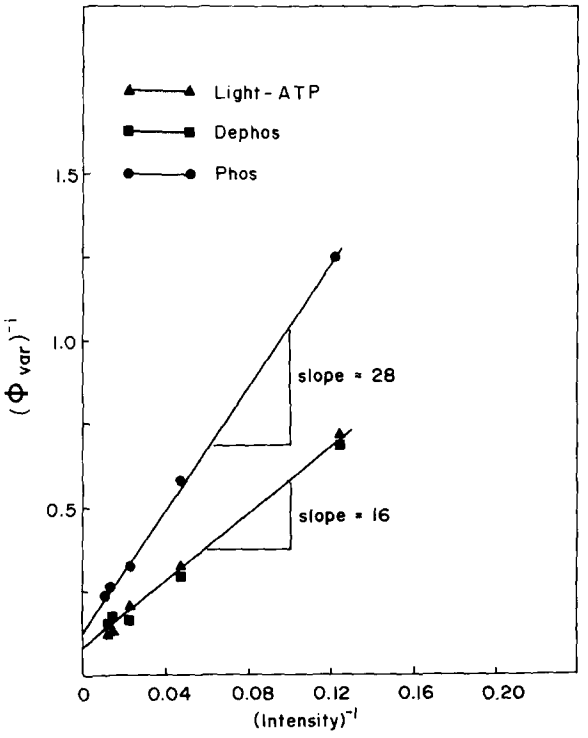

Fig. 7. Double-reciprocal plot of variable fluorescence against laser intensity. (For legend, see Fig. 6.)

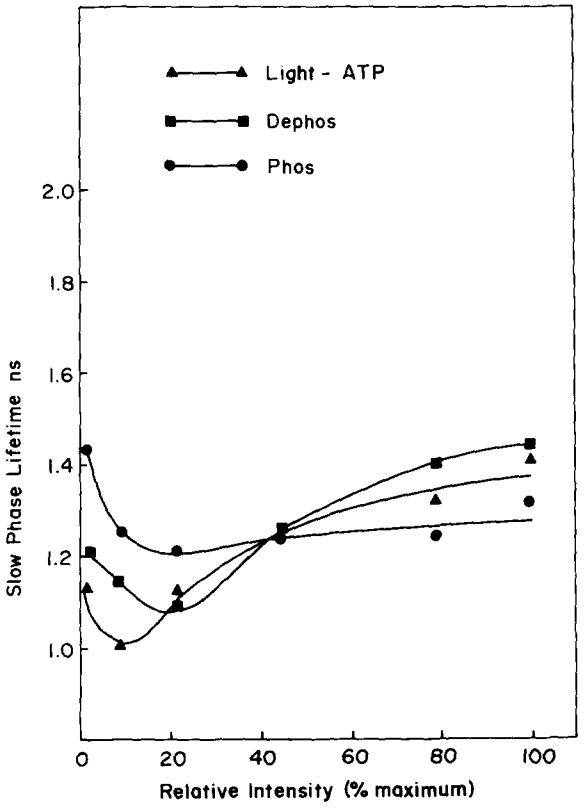

Fig. 8. Dependence of slow phase lifetime τ_s (ns) on laser intensity in light – ATP, dephosphorylated and phosphorylated membranes. (For legend, see Fig. 6.)

Discussion

The analysis of fluorescence decay kinetics should provide information on the mechanism of energy transfer and the functional organization of the light-harvesting system [24]. Room-temperature fluorescence decay, detected by single-photon counting, can be accounted for by three exponential components [8,9,25]. Nairn et al. [9] have adapted the tripartite model of Butler and coworkers [26,28] to hypothesize an explanation of these three components. The fastest phase (about 100 ps) is kinetically controlled by the decay processes of the Chl a_2 antenna. The middle phase (300–750 ps) is kinetically controlled by the decay of LHC II, through processes that are dominated by transfer to the Chl a_2 and PS I. The third, slow phase (1.1–2.0 ns) is controlled by the presence of Q^-, and its lifetime is affected by two factors. The first is the recombination of P-680$^+$ and reduced pheophytin (I^-), and the second is the rate of fluorescence quenching from the chlorophyll antenna. Gulotty et al. [25] reported similar components in the fluorescence decay of barley chloroplast membranes. However, they suggested an alternative interpretation: the fast (112 ps) lifetime component represents the decay of excited antenna chlorophylls which are quenched by or closely associated with either PS I or efficiently quenching PS II centers. The middle (380 ps) lifetime component represents either the decay of excited antenna quenched by PS II operating less efficiently or the transfer of excitation from less efficiently coupled antenna pigments. An origin for the slowest component (2.2 ns) was not specified, although various possibilities were suggested.

State 1–State 2 transitions [10,11] are an adaptive mechanism by which the photosynthetic membrane of higher plants can regulate the distribution of absorbed quanta between the two photosystems in response to changes in incident light quality.

Early speculation suggested that these transitions might be mediated through changes in Mg^{2+} concentration [29]. Recent developments, however, clearly indicate that in vivo state transitions are linked to the phenomenon of LHC II phosphorylation [12–21]. The data supporting this link have been recently reviewed [15,16]. The dynamic control of the protein kinase [13,30] reflects an autoregulatory mechanism within the chloroplast. Briefly, it has been demonstrated that phosphorylation decreases the interaction between PS II centers from that characteristic of α (matrix) kinetics to that of β (separate) kinetics [19]. These changes are associated with an increase in the absorption cross-section of PS I at the expense of PS II and an increase in the energy 'spillover' from PS II to PS I [31,32]. All of these changes are reversible within a half-time of 5–7 min in peas [21]. Observations suggesting the migration of LHC II from grana to stromal lamellae [33,34] have led to the development of a model to explain these effects [15]. This reversible migration is caused by a charge imbalance resulting from the phosphorylation [35,36], and the process is reversed by dephosphorylation. It is assumed in this model that the mobile LHC II functionally associates with PS I in the stromal lamellae to increase its antenna size. As yet there is only indirect evidence to support this claim [38].

Horton and Black [14,20] reported that the light-induced phosphorylation of LHC II caused a quenching of F_0 and F_{max} simultaneously so that F_v remained unaltered. Bennett et al. [18] and Kyle et al. [19], by contrast, have observed a specific quenching of F_{var}. This difference has important mechanistic implications for the role of LHC II and PS II in state transitions [20]. In an attempt to resolve this controversy we have measured fluorescence changes using a dual control system. Horton and Black [14,20] used a light – ATP control for their investigations to counter the photoinhibitory effects caused by exposure to light at room temperature. Kyle et al. [19] used only a dark + ATP control to counter possible 'high energy state' effects. The data we present (Tables I and II) show clearly that the fluorescence yield of the light – ATP membranes is greater at the F_0 and F_{max} levels than the dark + ATP control. This difference is possibly due to some photodestructive process not readily reversed in the dark under our conditions. Yet, the extent of variable fluorescence under each condition is the same, which suggests that no significant loss of photochemistry has occurred. Phosphorylation of the membranes decreased the F_0 yield, in comparison to that of the light – ATP membranes (Table I) but had no effect on the F_0 yield in comparison to the dark + ATP control. This observation could be interpreted as a phosphorylation-induced loss of F_0 yield, as reported by Horton and Black [14,20], which is countering the apparent increase in F_0 caused by exposure to light. Alternatively, the membrane phosphorylation may prevent the photoinhibitory effect seen under the light – ATP condition, as was suggested by Jursinic and Kyle [37], so that no increase in F_0 occurred. This second explanation is in part borne out by the finding that the F_0 yield in dephosphorylated membranes does not return to the light – ATP level but remains constant. Furthermore, it is quite clear from these data that F_v is quenched; the F_{max}/F_0 ratio falls to 3.0 in phosphorylated membranes, in agreement with Kyle et al. [19]. Dephosphorylation reverses this trend so that the F_{max}/F_0 ratio increases to 3.4. It is not clear why a significant loss of F_0 has been reported elsewhere [14,20], but the possibility of variations in biological samples or experimental technique needs to be explored further [20]. In addition to a quenching of ϕ_s ($\equiv F_{var}$) we observe that phosphorylation affects ϕ_m (Table II and Fig. 4). This increase in middle phase yield could arise from less efficient energy transfer between LHC II and Chl a_2 [9] or from an increase in energy transfer to less efficient reaction centers [25], possibly β-centers. Either explanation is consistent with the pigment bed changes implicit in the proposed model [15]. Decreases in ϕ_t reported here and elsewhere [19] are indicative of less PS II activity following phosphorylation. This interpretation is supported by direct measurements of PS II activity [21,38], which is also decreased in phosphorylated membranes. In addition to changes in the fluorescence yield, phosphorylation alters the intensity dependence of the fluorescence (Figs. 3–7). These data indicate a decreased antenna size for the PS II, which is reversed upon dephosphorylation. Such observations are fully consistent with data derived from analysis of low-temperature (77 K) fluores-

cence [31,32], the reversible shift from α- to β-centers [19] and decreased PS II activity [21,38]. The lifetimes of the two faster components are relatively constant. It is apparent from the data of Table I and II and Fig. 8 that the slow phase lifetime of phosphorylated membranes is independent of reaction center redox state, in that the lifetimes of F_0 and F_{max} are the same (1.4 ns). Under other membrane conditions the transition from F_0 to F_{max} causes the slow phase lifetime to increase (1.1 to 1.5 ns). This difference is not the result of exposure to light, because it is not observed in either dephosphorylated or light – ATP membranes. Nor is it likely that the anomaly in lifetime arises from the presence of 'free' or disconnected LHC II, because this should exhibit a lifetime greater than 3 ns [39]. The absence of this longer lifetime component indicates that the migrating LHC II is quenched by some species or mechanism, possibly PS I. A possible explanation can be deduced from Fig. 8, which indicates that the slow phase lifetime is not homogeneous; similar data were reported by Haehnel et al. [8]. One explanation for this might be that the slow phase at F_0, where Q is fully oxidized, does not arise from the back-reaction of $P\text{-}680^+$ and I^-, this recombination being significant only at higher intensities where Q becomes at least partially reduced. Alternatively, the heterogeneous slow phase might arise from α- and β-centers [5,6], so that changes in τ_s would reflect the different kinetics and/or efficiency of these centers. The earlier slow phase with shorter lifetime might arise from α-centers, while the slower β-centers would contribute only at higher intensities. Because phosphorylation causes an increase in the amount of β-centers (Ref. 21 and Melis and Haworth, unpublished observation), the α-center contribution to the slow phase should be less significant and the lifetime should remain constant.

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