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THYLAKOID MEMBRANE PROTEIN PHOSPHORYLATION LEADS TO A DECREASE IN CONNECTIVITY BETWEEN PHOTOSYSTEM II REACTION CENTERS

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The room-temperature fluorescence induction transients from stroma-free chloroplast membranes (in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea) have been analyzed to determine the effects of membrane protein phosphorylation on the connectivity between Photosystem (PS) II centers. Chloroplast membranes which have been incubated in the light with ATP exhibit: (1) a decrease in the variable fluorescence as a function of the initial fluorescence, (2) a shift from a sigmoidal to an exponential fluorescence induction curve, and (3) a reduced amount of the fast (α) component of the induction transient. These phenomenona are completely reversible by dark incubation of the samples (leading to protein dephosphorylation). We conclude that connectivity between PS II centers is reduced as a function of thylakoid membrane protein phosphorylation. This may in turn be the mechanism which increases the amount of absorbed excitation energy available to PS I.

Introduction

The transfer of energy between PS II units was hypothesized by Joliot and Joliot [1] from the analysis of room temperature fluorescence induction transients in the presence of DCMU. The induction curve was shown to be sigmoidal rather than exponential; this was explained by proposing that energy transfer could occur from the PS II antennae serving a closed PS II reaction center to those of an open PS II center. Further analysis indicated that the area over the induction curve was biphasic and consisted of a fast, nonexponential component (α), and a slower, exponential component (β) [2,3]. The kinetics of cytochrome *c*-550 and *a*-320 changes (due to the reduction of the primary stable electron acceptor of PS II) observed on the onset of illumination are also biphasic and virtually identical to the area over the fluorescence induction transient [4]. This suggests the existence of two components in the primary photochemistry of PS II at room temperature. On the basis of these kinetics, the α-component consists of connected PS II centers (matrix model; energy transfer between PS II centers does occur), and the β-component with separate units (separate package model; energy transfer between PS II centers does not occur). It has been suggested that α- and β-centers may, in fact, be distinct physical entities [2,5].

Divalent cations have been shown to have a profound effect on excitation energy distribution between the two photosystems [6]. In the absence of cations, absorbed excitation energy is preferen-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)methylglycine PS, photosystem; LHC_{II}, light-harvesting chlorophyll-protein complex of PS II; Q, primary stable electron acceptor of PS II; Chl, chlorophyll; F_m, maximal level of fluorescence; F_0, initial level of fluorescence.

tially distributed in favor or PS I and energy transfer between PS II centers is reduced. Melis and Homann [3] have presented evidence indicating that the α-component of the room-temperature fluorescence transient, but not the β-component, is affected by Mg^{2+}. This suggests that under cation-deficient conditions, PS II reaction centers act more like a separate package model and the energy transfer between PS II centers is reduced.

Bennett [7] has demonstrated a light-induced, covalent modification of chloroplast membranes by protein phosphorylation. This results in a shift in the distribution of absorbed excitation energy in favor of PS I as indicated by an increase in the fluorescence emission at 735 nm with respect to that at 685 nm (F_{735}/F_{685}) measured at 77 K [8]. The principal phosphorylated protein is the light-harvesting chlorophyll-protein complex serving PS II (LHC_{II}). LHC_{II} is required for the regulation of excitation energy transfer [9]. A model has recently been proposed which involves the phosphorylation of LHC_{II} to provide a physiological explanation for the control of the distribution of excitation energy between the two photosystems [10].

In this report, we present evidence indicating that the proportion of α- and β-components of the room-temperature fluorescence kinetics are affected by LHC_{II} phosphorylation. That is, phosphorylation leads to a reversible increase in the separate package, or β-component, and a decrease in the matrix, or α-component of the fluorescence induction curve. This results in a net decrease in PS II-PS II energy transfer. In parallel studies we have verified that under our experimental conditions, ^{32}P label is reversibly incorporated into LHC_{II} [8].

Materials and Methods

Chloroplasts were prepared from 20–24-day-old pea seedlings by grinding the leaves in 15 mM Tricine (pH 7.8) buffer containing 0.4 M sorbitol and 10 mM NaCl. The homogenate was filtered through 4 and then 12 layers of cheesecloth and centrifuged for 5 min at $5000 \times g$. This crude pellet was washed once in 10 mM Tricine (pH 7.8) containing 10 mM NaCl and 5 mM $MgCl_2$ and resuspended to a concentration of 100 μg Chl/ml in 10 mM Tricine (pH 7.8) containing 0.1 M sorbitol, 10 mM NaCl and 5 mM $MgCl_2$. The samples were placed in a glass water bath for 5 min to come to room temperature. ATP was then added to a concentration of 200 μM and phosphorylation commenced at the onset of illumination with white light (500 $\mu E/m^2$ per s). To prevent dephosphorylation in phosphorylated samples, NaF was added to a concentration of 10 mM to inhibit the membrane-bound phosphatase [11]. Phosphorylated samples are defined as those incubated for 15 min light followed by 15 min dark in the presence of NaF, dephosphorylated samples by 15 min light followed by 15 dark before addition of NaF, and nonphosphorylated samples by the full 30 min in the dark before addition of NaF. For measurements made in the absence of Mg^{2+}, the samples were washed three times in Mg^{2+}-deficient resuspension buffer containing 10 mM NaF after the phosphorylation/dephosphorylation.

For fluorescence measurements, the chloroplast membrane samples were diluted to 5 μg Chl/ml in the resuspension buffer containing 5 μM DCMU. The fluorescence induction transients were recorded on a Nicolet Explorer II digital recording oscilloscope. Broad-band blue light (Corning 4-96) was the actinic source, and fluorescence was measured by a photodiode 90° from the incident light using a Corning 2-64 red filter. The data for each induction transient were transferred from the oscilloscope, and processed by an HP-85 minicomputer, using analyses based on the method of Melis and Homann [3] and programs developed in our own laboratory.

Results

The magnitude of the variable component of room-temperature fluorescence in the presence of DCMU is reduced as a result of membrane protein phosphorylation. This reduction appears to be independent of any membrane photodestruction, since the large variable component is restored upon dephosphorylation (Fig. 1). It is clear from the induction transients that it is primarily the maximal level of fluorescence (F_m) rather than the initial level of fluorescence (F_0) that is affected by protein phosphorylation under our conditions. In

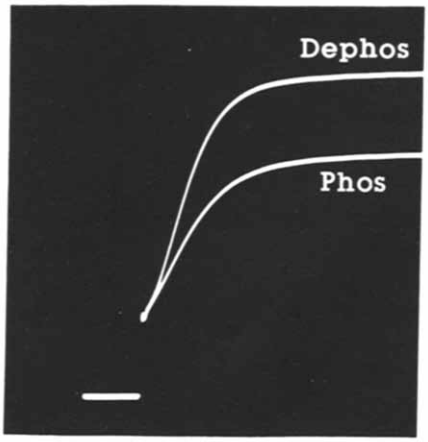

Fig. 1. Fluorescence induction transients in the presence of DCMU of phosphorylated (Phos) and dephosphorylated (Dephos) membranes. Samples were dark adapted for 5 min prior to the addition of 5 μM DCMU and measurement.

addition to the decrease in F_m, the shape of the induction curve in the presence of DCMU is altered. Nonphosphorylated and dephosphorylated membranes had characteristically sigmoidal fluorescence induction curves, whereas phosphorylated membranes had more exponential induction curves.

The kinetics of the increase of variable fluorescence during an induction in the presence of DCMU is a measure of the rate of closure of the PS II reaction centers (reduction of Q, the primary stable electron acceptor of PS II). The growth in the area over the fluorescence induction curve has been plotted as a function of the variable fluorescence for nonphosphorylated, phosphorylated and dephosphorylated membranes (Fig. 2). According to Melis and Schreiber [4], the initial positive deflection from a slope of unity represents the α-centers (matrix-type centers) and the negative deflection at higher levels of variable fluorescence results from the presence of β-centers (separate centers). The dashed line (slope of unity) in Fig. 2 represents the expected result if all PS II centers behaved completely as separate units. The data in Fig. 2 indicate that as a result of phosphorylation there is less connectivity between the PS II centers, and that this phenomenon is reversible when samples dephosphorylate in the dark (in the absence of NaF).

If the growth in the area over the induction curve is plotted as a function of time, a measurement for the closure of PS II traps is obtained (Fig. 3, solid line). These data can be deconvoluted mathematically using a plot of ln(1 − area growth) vs. time. This plot indicates that the trap closure occurs in a biphasic manner [2] (Fig. 3, dotted line). The two phases are defined by intersecting straight lines from which kinetic constants can be obtained. These data indicate a reduction in the fast component (α-centers) as a result of phosphorylation; this is reversible on dephosphorylation. With the α- and β-components determined from Fig. 3, new area growth curves were computed to quantify the relative contributions of α- and β-components in all samples (Fig. 4). These data clearly indicate a reversible change in propor-

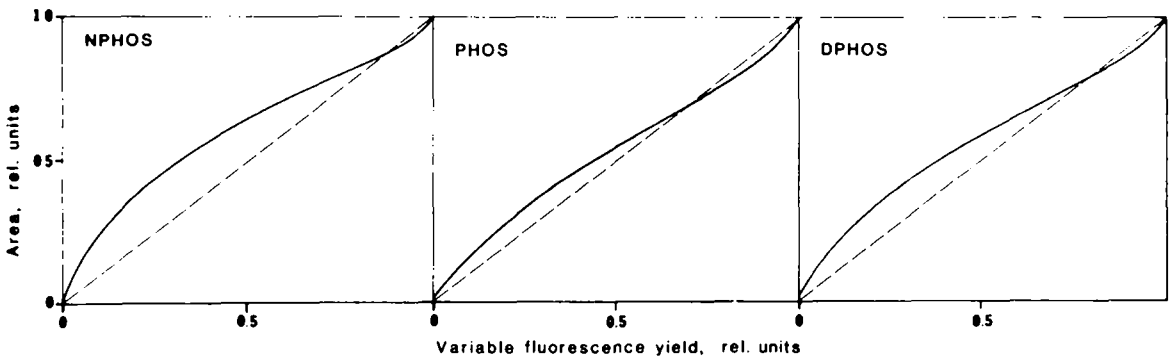

Fig. 2. Fraction of closed PS II centers (area growth) vs. the variable yield fluorescence for nonphosphorylated (NPHOS), phosphorylated (PHOS) and dephosphorylated (DPHOS) membranes. Theoretical predictions for a separate package model (zero connectivity) are given by the dashed lines.

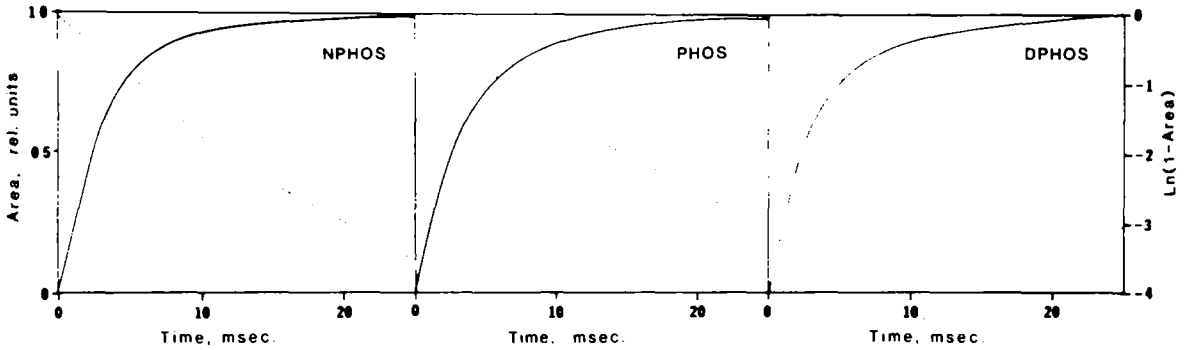

Fig. 3. Kinetics of the fast and slow components of the PS II center closure in nonphosphorylated, phosphorylated and dephosphorylated membranes. The time course is shown for the PS II unit closure (————) and for the logarithmic plot of 1 − area (· · · · · ·). Abbreviations as in Fig. 2.

tion of α- and β-components as a result of membrane protein phosphorylation.

The relative proportion of α- and β-components of the fluorescence induction curve has been shown to be affected by the presence or absence of divalent cations. In the absence of Mg^{2+}, F_m decreased without appreciably changing F_0, the shape of the induction curve was shifted from sigmoidal to exponential, and the proportion of the α-component was significantly reduced [3]. Since it has been proposed that protein phosphorylation mimics State II transitions [10] similar to that in the absence of Mg^{2+}, we have compared the effects of membrane protein phosphorylation to the effects of the absence of cations on room-temperature fluorescence. The area growth as a function of variable fluorescence in cation-depleted, nonphosphorylated membranes generated an approximately linear relationship, indicating no connection between PS II reaction centers (Fig. 5). The closure of the reaction centers as a function of time occurred as an exponential function (no longer biphasic) and it was only the slow component (β) which remained (Figs. 6 and 7). These results with cation-depleted membranes were identical regardless of the state of phosphorylation of the membranes (data with phosphorylated and dephosphorylated membranes not shown). Therefore, although membrane protein phosphorylation reduces the connectivity between PS II centers, a cation-deficient medium completely abolishes any connectivity as judged by room-temperature fluorescence transients.

The effect of cation depletion upon features of

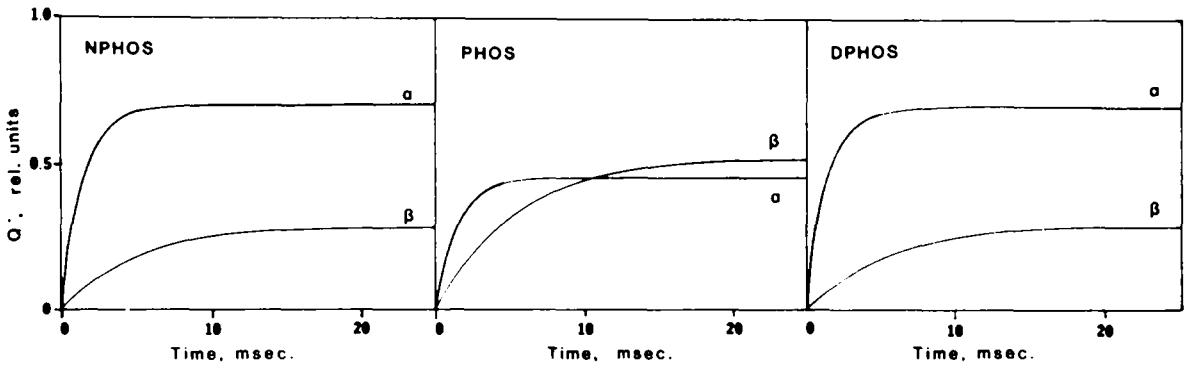

Fig. 4. Normalized contributions of the α- and β-components of the PS II center closure for nonphosphorylated, phosphorylated and dephosphorylated membranes. α- and β-component rise curves are calculated from the components of the ln(1 − area) curves in fig. 3, with $\beta = 1 - \alpha$. Abbreviations as in Fig. 2.

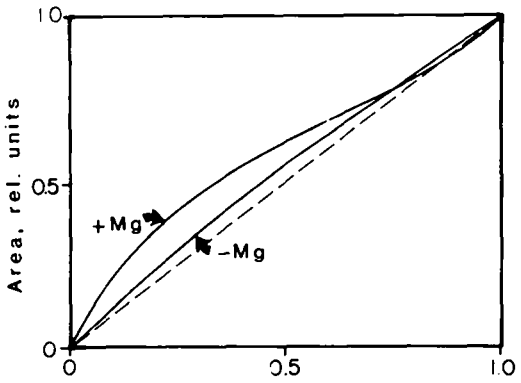

Fig. 5. The effect of Mg^{2+} on the kinetics of PS II center closure in nonphosphorylated membranes. Fraction of closed PS II centers as a function of variable fluorescence yield in the absence or presence of 5 mM $MgCl_2$. A plot for zero connectivity is given by the dashed line.

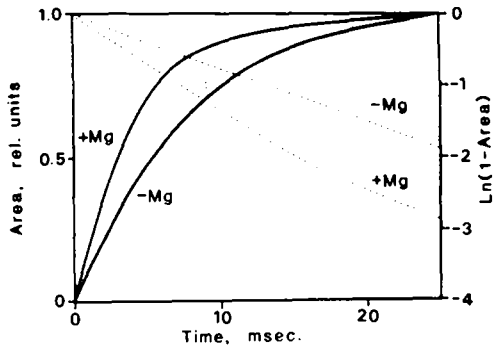

Fig. 6. The effect of Mg^{2+} on the kinetics of the fast and slow componets of PS II center closure in nonphosphorylated membranes. Experiments were carried out in the absence or presence of 5 mM $MgCl_2$. Time course is shown for the PS II center closure (————) and logarithmic plot of 1 − area (· · · · · ·).

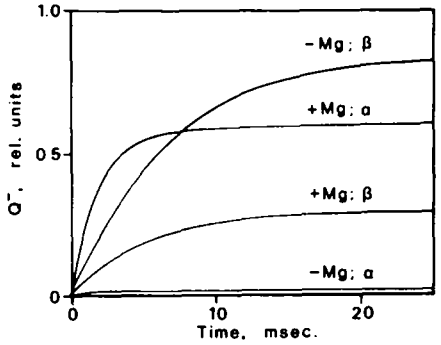

Fig 7. The effect of Mg^{2+} on the normalized contributions of the α- and β-components of the PS II center closure. Curves are shown for the absence or presence of 5 mM $MgCl_2$. α- and β-component rise curves are calculated from the components of the ln (1 − area) curves given in Fig. 6 with $\beta = 1 - \alpha$.

the fluorescence induction transient are substantially different from those imposed by protein phosphorylation (Table I). Cation depletion caused a strong reduction in F_m and a slight decrease in F_0, whereas protein phosphorylation reduced F_m but slightly increased F_0. The α-component eliminated in cation-depleted samples but was reduced by approx. 35% as a result of 15 min of phosphorylation. The half-times of the α- and β-induction components (Table I) did not change to a large extent in either situation. In other experiments (data not presented) longer periods of protein phosphorylation led to a further decrease in the percentage of the α-component to nearly zero. However, prolonged periods of exposure to

TABLE I

PARAMETERS FOR THE ROOM-TEMPERATURE FLUORESCENCE INDUCTION IN THE PRESENCE OF DCMU

The top half of the table indicates the effect of phosphorylation on these parameters. Data given in the bottom half of the table were from nonphosphorylated chloroplasts which were washed free of Mg^{2+} (i.e., unstacked). 5 mM $MgCl_2$ was added back to these samples and induction transients were observed 5 min after this readdition. Values are given for the initial fluorescence (F_0), maximal fluorescence (F_m) and variable fluorescene ($F_v = F_m - F_0$) as a function of F_0, as well as the extent of the α- ($F_{v\alpha}$) and β-components ($F_{v\beta}$) and the half-time for α- (T_α) and β-components (T_β) in ms. $F_{v\beta}$ is given as $1 - F_{v\alpha}$.

	F_0	F_m	F_v/F_0	$F_{v\alpha}$	T_α	$F_{v\beta}$	T_β
Nonphosphorylated	364 ± 15	1444 ± 74	2.97	0.71 ± 0.03	2.27 ± 0.22	0.29	6.80 ± 0.31
Phosphorylated	427 ± 19	1002 ± 35	1.35	0.46 ± 0.02	2.06 ± 0.21	0.53	7.52 ± 0.33
Dephosphorylated	430 ± 24	1331 ± 23	2.10	0.70 ± 0.01	1.82 ± 0.02	0.30	7.64 ± 0.48
Minus Mg	550 ± 24	1200 ± 17	1.18	0	0	1.00	6.51 ± 0.21
Readdition of Mg	656 ± 5	1570 ± 10	2.39	0.65 ± 0.02	2.32 ± 0.03	0.35	5.64 ± 0.25

light at room temperature led to an irreversible decrease in the variable fluorescence.

Discussion

The distribution of absorbed excitation energy between PS I and PS II in chloroplast thylakoids is influenced by the cation concentration of the suspension medium [6,12]. It has recently been demonstrated that a further regulation is brought about by phosphorylation of thylakoid membrane proteins [8,10]. The manifestation of this effect is observed in physiological concentrations of cations. This work is directed at understanding the underlying mechanisms mediating the change in light-harvesting properties of thylakoid membranes.

Our data indicate that protein phosphorylation results in a decrease in the α-component of the room-temperature fluorescence induction transient (Table I) and therefore a decrease in the connectivity between PS II centers; this effect is reversible upon dephosphorylation. The effect of Mg^{2+} depletion, like phosphorylation, also decreased the α-component, however, cation depletion totally abolished the α-component whereas phosphorylation for 15 min only reduced the α-component by about 35%. Melis and Homann [3] also noted a decrease in the α-component, in cation-deficient chloroplasts, although their preparations were not so extensively salt depleted as to lose the α-component totally.

There are two major points of significance in the interconversion of the α- and β-components by protein phosphorylation; the changes relate to: (1) the underlying basis for these components, and (2) the mechanism by which physiological control of light harvesting is expressed. With respect to the first idea, there have been two divergent concepts of the nature of α- and β-components in fluorescence induction transients. Melis and Duysens [2] and Thielen and Van Gorkom [5] have suggested that these arise from two distinct types of PS II centers. Alternatively, Butler [13] has proposed that the α-component is simply a measure of connectivity between PS II units, and it is therefore not necessary to require the existence of two fundamentally different types of reaction centers. The rapid conversion of the α-component into the β-component during protein phosphorylation strongly favors the latter concept; i.e., it is a change in the 'connectivity' in the connected package model which is affected by membrane protein phosphorylation. Implicit in this interpretation is the fact that there does not seem to be any realistic suggestion by which the phosphorylation could make a fundamental change in the reaction center per se.

A second point concerning the interconversion of α- and β-components via protein phosphorylation relates to the mechanism by which the phosphorylation controls distribution of absorbed excitation energy between the two photosystems. Approx. 50% of the white light absorbed by the chloroplasts is collected by the light-harvesting complex serving PS II (LHC_{II}). It is the polypeptides of this complex which are the predominant phosphorylated protein species in the thylakoid [8,11]. It has been shown that the LHC_{II} is required to observe both cation-induced changes in energy distribution between PS II and PS I, as well as PS II-PS II energy transfer [14,15]. A photon absorbed within a specific LHC_{II} can result in energy transfer to a PS II or a PS I center, or transfer to another LHC_{II}. The latter event is equivalent to maintaining the excitation energy available to the PS II pigment bed. When a process such as protein phosphorylation restricts energy transfer among PS II units, as shown in this work, the effective result is an increased probability of other pathways of energy transfer, in this case especially an increased sensitization of PS I. This is, obviously, a 'State II' adaptation. We will demonstrate in a companion paper [16] that analysis of fluorescence transients at 77 K via the bipartite models of photosynthesis verifies this concept.

The changes in energy distribution brought about by protein phosphorylation show many similarities to those induced by cation depletion of thylakoids. We emphasize that the mechanisms controlling both may have similar underlying bases, relating to the membrane surface-exposed segment of LHC_{II} [15,17]. There are, however, subtle but important differences. Firstly, the in vivo chloroplast Mg^{2+} concentrations appear never to fall below a level which would fully saturate the energy distribution response [18]. The protein phosphorylation functions, therefore, in a fashion which

only requires cations as a prerequisite for maintenance of normal thylakoid activity. Secondly, the phosphorylation-related changes in the pigment bed are likely to involve less drastic membrane alterations than cation depletion. We will demonstrate in a subsequent publication that protein phosphorylation does not lead to grana destacking as does cation depletion. Alterations within the pigment bed leading to decreased PS II-PS II transfer arise from subtle changes in the functional organizations of LHC_{II} relating to its location within grana or stroma lamellae [20].

As a final point, we should note that Horton and Black [19] have also analyzed fluorescence induction transients in phosphorylated membranes. Their treatment conditions are different from ours, as are some aspects of their data. While both laboratories observed a quenching of F_m, their data indicate a static quenching (i.e., proportional reduction of both F_m and F_0). This would be equivalent to changes in the properties of the chloroplast pigment bed which could range from an alteration of the fluorescence rate constant to a modification of the direct sensitization of PS I. Our data are clearly different; the selective decrease in variable fluorescence observed in this work indicates a dynamic quenching which we have proposed to be related to altered energy transfer among PS II centers. We cannot determine the reason for differences in the two sets of data (Ref. 19 and this work) at the present time. In support of our own observations, we note that all experiments were done to show reversibility of all phenomena, i.e., in nonphosphorylated, then phosphorylated and finally dephosphorylated samples. In addition, fluorescence analysis at 77 K using a markedly different data evaluation system provided additional evidence for a dynamic quenching process relating to energy coupling in PS II centers as well as a change in the α-component [16]. It is possible that the experimental conditions utilized by Horton and Black [19] preferentially led to the latter change.

Acknowledgement

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