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THE EFFECT OF REDOX POTENTIAL ON THE KINETICS OF FLUORESCENCE INDUCTION IN PEA CHLOROPLASTS

II. SIGMOIDICITY

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The shape of the fluorescence induction curve in chloroplasts inhibited by 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea has been determined at different redox potentials. At −10 mV a monophasic and sigmoidal curve is seen which is transformed into an exponential curve when the potential is poised at −150 mV. At this potential, the quencher with high midpoint, Q_H, is reduced but that with low midpoint, Q_L, is oxidized. Thus, a sigmoidal induction is observed during photoreduction of Q_L and Q_H but photoreduction of Q_L proceeds with exponential kinetics. A correlation between the relative proportions of Q_L and Q_H observed in redox titration and the sigmoidicity of induction is also seen upon depletion of Mg^{2+} and after alkalinization to pH 9.5. Several models are discussed to explain the relationship between Photosystem II interactions and Q heterogeneity.

Introduction

In the presence of the inhibitor DCMU, the rise in chlorophyll fluorescence observed upon illumination reflects the photochemical reduction of Q, the primary electron acceptor of PS II [1]. This fluorescence induction curve does not show the simple first-order kinetics expected for a single photochemical electron transfer. In fact, it consists of two phases; a fast sigmoidal phase and a slow exponential one. Investigation of the fast phase had led to the general acceptance that sigmoidicity is due to energy transfer between PS II units [2–6]. This theory has been more precisely defined in terms of a 'statistical pigment bed' in which a domain of PS II reaction centres is visualized as being randomly embedded in an array of light-harvesting chlorophylls; PS II units 'interact' by means of energy transfer into (and out of) the PS II centres from (and to) the light-harvesting chlorophylls [7,8]. In qualitative terms, what this means is that a photon arriving at a closed reaction centre can, by energy transfer, find its way to an open one; hence a 'lag' is observed in the rise in fluorescence vs. time. Looked upon in another way, the fluorescence yield also 'lags' behind the amount of Q reduced compared to that expected for a simple linear relationship between $[Q^-]$ and F_v; a plot of $[Q^-]$ vs. F_v is therefore hyperbolic. In recent years photosystem models based on energy transfer between various pigment sub-sets have been described [7–10]. It should be emphasized, however, that sig-

Abbreviations: PS II, Photosystem II; Q_L, fluorescence quenching component with E_{m_7} about −250 mV; Q_H, fluorescence quenching component with E_{m_7} about −20 mV; F_0, minimum level of fluorescence when Q is oxidized; F_m, maximum level of fluorescence when Q is reduced; F_i, initial fluorescence level, equal to F_0 when Q is fully oxidized; F_v, $F_m - F_i$, variable fluorescence; LHCP, light-harvesting chlorophyll protein; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxymethyl)methylglycine; Caps, cyclohexylaminepropanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid.

moidal induction kinetics can be predicted by models other than these [3]. Notably, other models have proposed that PS II trap closure requires more than a single photon [11–13].

These alternative models are of particular interest in that Q does not appear to be a single redox component. Of particular interest are the Q components with E_{m_7} at approx. –250 mV (Q_L) and 0 mV (Q_H) seen in redox titration of fluorescence yield [14–20] and the PS II-related *P*-518 absorbance change [21]. Some observations suggested that these quenchers may be segregated in different parts of the membrane system, with Q_H being involved in the slow exponential phase of induction and being confined to stromal membranes [16,19,22]. However, recent data showed that this phase can be eliminated by prior potentiometric reduction at redox potentials far more positive than that of Q_H [23,24], thus opening up the possibility that Q_H and Q_L exist together in the same reaction centre. This has been argued for by other workers [17]. A model in which Q_L and Q_H are both electron acceptors in a given PS II unit could generate sigmoidal induction kinetics provided that certain conditions regarding the rate constants of, for example, $Q_L Q_H \rightarrow Q_L Q_H^-$ and $Q_L Q_H^- \rightarrow Q_L^- Q_H^-$ are fulfilled (Hipkins, M., personal communication).

It was therefore decided to investigate the effect of poising the redox potential of Q at different values prior to illumination on the sigmoidal kinetics of fluorescence induction. Because of the complexity involved, it is necessary to actually titrate the induction curve shape rather than just examine two or three curves at chosen potentials, as in earlier studies [16,25]. The relationship between Q_L/Q_H and sigmoidicity can also be tested by investigating situations in which sigmoidicity is absent or reduced (e.g., after depletion of divalent cations). Besides establishing the feasibility of two-photon PS II models, this study would conversely allow certain conclusions to be made about Q organization and distribution in terms of the statistical pigment bed scheme.

Materials and Methods

Pea chloroplasts were isolated as described previously [26]. Depletion of divalent cations was performed by resuspension in a large volume of medium containing 0.1 M sucrose, 10 mM NaCl and 10 mM Mops, pH 7.0, and centrifuging at 3000 × *g* for 5 min. Pellets were then resuspended in the usual resuspension medium ±$MgCl_2$. Redox potentiometry followed procedures published earlier [16,19,23] except that extra care was given to the choice and concentration of redox mediators, some of which can act as electron acceptors or quenchers [16,18]. The reaction medium for all experiments contained 0.1 M sucrose, 10 mM NaCl, ±5 mM $MgCl_2$ and 10 mM Tricine, adjusted to pH 7.8 with NaOH. Chloroplasts were diluted to a concentration of 15 μg chlorophyll/ml. Measurement of chlorophyll fluorescence was made as described previously [23] and induction curves were stored by a Datalab DL406 Analyser equipped with a DL4016 Sweep Timer set at 800 μs/address and a DL403 Display. Induction was initiated by the opening of a Uniblitz electronic shutter with an opening time of 0.6 ms and stirring was discontinued during measurement. Analysis of induction curves was performed using programmes and routines provided on-line by a DL417 Function module.

Results

If Q is progressively reduced in chloroplasts prior to recording an induction curve, the ratio F_v/F_m will decline from its maximum level close to 0.7 (Q fully oxidized) to 0 (Q fully reduced). Fig. 1 shows a redox

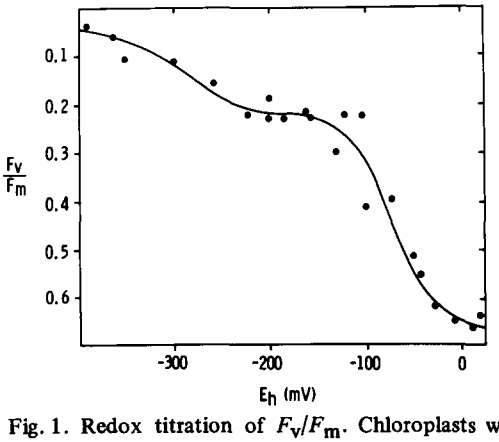

Fig. 1. Redox titration of F_v/F_m. Chloroplasts were incubated for 10 min in darkness at different redox potentials. DCMU (5 μM) was then added and fluorescence induction curves recorded as described in the text. The redox mediators were 1,4-naphthoquinone, indigotetrasulphonic acid, 2-hydroxy-1,4-naphthoquinone and anthraquinone disulphonate.

titration of F_v/F_m revealing the presence of the two components of Q, previously characterized by titration of the fluorescence yield under low-intensity illumination and called Q_L and Q_H [16]. Here, Q_L and Q_H have $E_{m_{7.8}}$ values of −290 and −75 mV, respectively. As discussed previously [23], reductive titration of Q in complete darkness is very difficult due to extremely sluggish redox equilibration, so that the data in Fig. 1 was obtained oxidatively.

Having shown that F_v/F_m can be titrated in such a way as to show the presence of the two quenchers, the kinetics of induction were investigated. In Fig. 2 are shown two induction curves recorded at −10 mV when Q is fully oxidized and at −150 mV when Q_H is reduced but Q_L oxidized. At −10 mV it should be noted that the slow phase has already been eliminated [23]. A typical sigmoidal induction is seen at this redox potential, but at −150 mV sigmoidicity appears to have been lost. This is best shown in a plot of F_v vs. area growth above the induction curve (Fig. 3). At −10 mV this plot gives a characteristically 'hyperbolic' curve, whilst at −150 mV a linear plot is obtained, indicating a directly proportional relationship between $[Q^-]$ and fluorescence yield that results during an exponential induction. The shift away from sigmoidicity is a gradual one as the redox potential is progressively lowered; no further change in the shape of the induction curve took place as Q_L was progressively reduced. This is shown by data in Fig. 4, in which the half-time for fluorescence induction is plotted vs. redox potential. It was found that the change in induction kinetics closely follows potentiometric reduction of Q_H, but is unchanged during Q_L reduction.

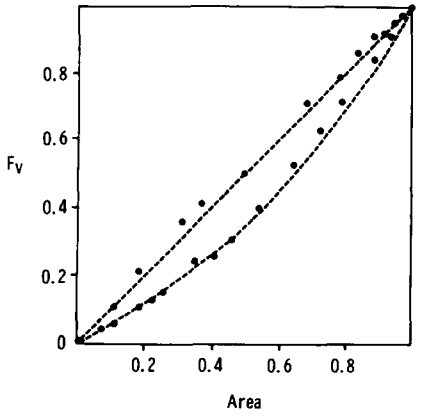

Fig. 3. F_v vs. area plot for the induction curves in Fig. 2. Linear plot is for data at −150 mV and curved plot at −10 mV. Dotted lines correspond to p values of 0 and 0.48 where p is given by $F_v = (1-p)(1-[Q])/1-p(1-[Q])$ as described in Ref. 5.

The above data are consistent with the notion that sigmoidicity is related to the presence of oxidized Q_L and Q_H prior to induction. However, of course, in a model in which sigmoidicity is explained by sharing of excitation by PS II centres in a statistical pigment bed, sigmoidicity is also expected to diminish as Q is

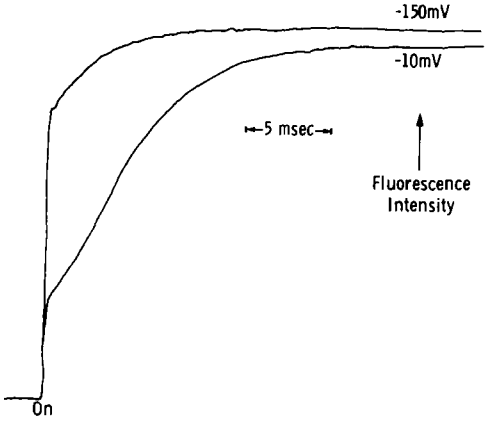

Fig. 2. Induction curves at different redox states of Q. Induction curves were recorded at −10 mV (Q_H and Q_L fully oxidized) and −150 mV (Q_H reduced, Q_L oxidized) as described for Fig. 1.

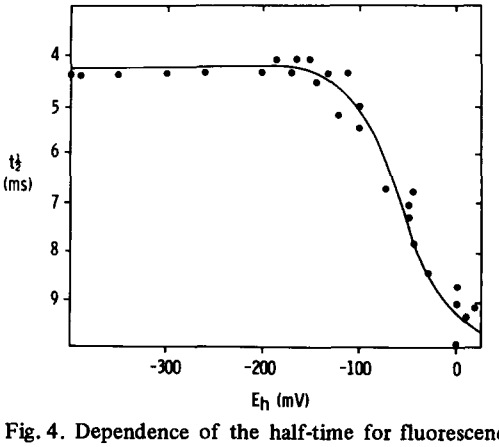

Fig. 4. Dependence of the half-time for fluorescence induction on redox potential. Data were obtained as in Fig. 1. $t_{1/2}$ is being used as an estimate of the amount of sigmoidicity.

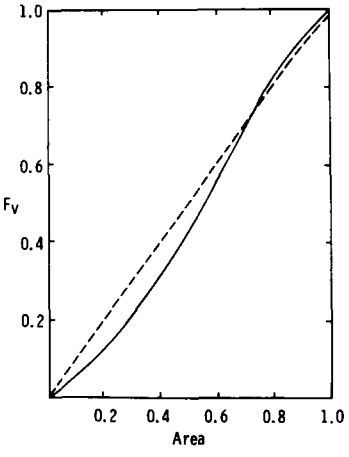

Fig. 5. F_v vs. area plots for induction curves recorded for chloroplasts washed and resuspended in an Mg^{2+}-free medium (- - - - -) and one containing 3 mM $MgCl_2$ (———). Induction curves were recorded under aerobic conditions, in the presence of DCMU.

chemically reduced before illumination. An alternative way of probing this relationship is to examine situations in which alteration in sigmoidicity is induced by perturbation of the normal membrane environment. This approach complements an earlier investigation in which it was shown that in developing, agranal chloroplasts, Q_L is absent [19] and fluorescence induction is non-sigmoidal [27].

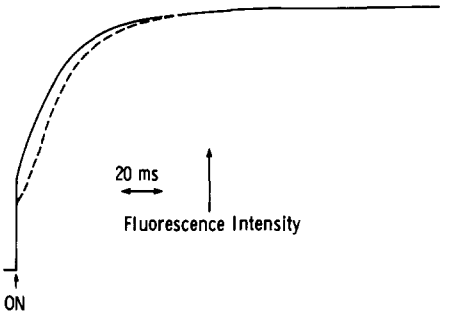

Fig. 7. Fluorescence induction at pH 8 (- - - - -) and pH 9.5 (———). Induction was carried out under aerobic conditions after 5 min dark incubation at the appropriate pH. Media were buffered with 10 mM Tricine (pH 8.0) and 5 mM Tricine and 5 mM Caps (pH 9.5).

Washing and resuspension of chloroplasts in a medium containing no divalent cations and 5–10 mM NaCl results in a large decrease in fluorescence yield [28]. Induction curves for these chloroplasts are non-sigmoidal [5,29]. Fig. 5 shows the characteristic F_v vs. area plot which is linear in the absence of Mg^{2+} and non-linear in its presence. A redox titration of Q in these chloroplasts is shown in Fig. 6. Depletion of

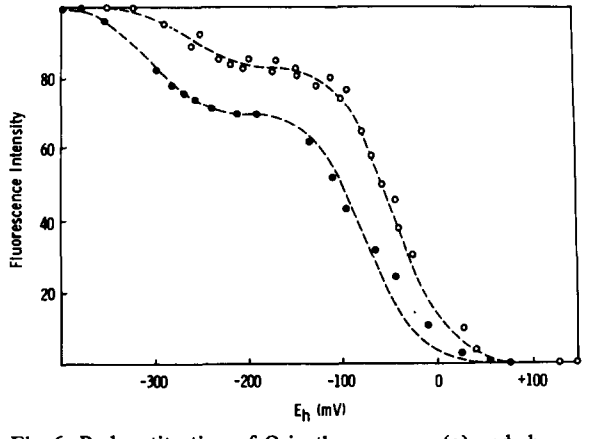

Fig. 6. Redox titration of Q in the presence (•) and absence (○) of Mg^{2+}. Chloroplasts were prepared as described in the text and in Fig. 5. Redox titration of the level of fluorescence under continuous low-intensity light was performed as previously described [16,19,20] using the redox mediators of Ref. 20. Dotted lines are Nernst equations (n = 1) with E_{m_8} at −300 mV (30%) and −80 mV (70%) with Mg^{2+}, and −265 mV (16%) and −45 mV (84%) without Mg^{2+}.

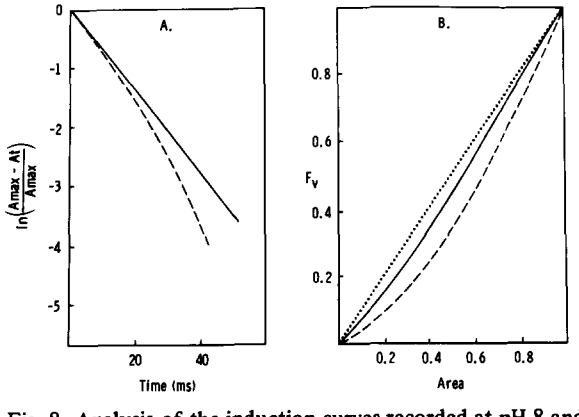

Fig. 8. Analysis of the induction curves recorded at pH 8 and pH 9.5. (A) Semilogarithmic plots of the area growth above the induction curve at pH 8 (- - - - -) and pH 9.5 (———). (B) F_v vs. area plots at pH 8 (- - - - -) and pH 9.5 (———) A linear (p = 0) line is also shown (· · · · · ·). The analysis was based on the method of Melis and Homann [29] modified as in Ref. 33. Essentially, the slow β phase has been subtracted prior to the analyses shown in this figure.

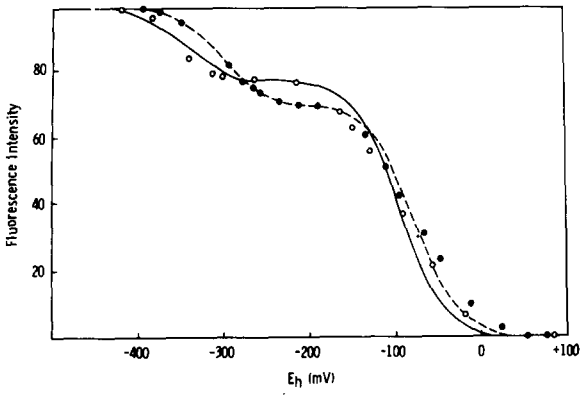

Fig. 9. Redox titration of Q at pH 8 (•) and pH 9.5 (○). Titrations were performed as in Fig. 6. Nernst equations (n = 1) are shown for E_{m_8} = −300 mV (30%) and −80 mV (70%), and $E_{m_{9.5}}$ = −360 mV (22%) and −85 mV (78%).

Mg^{2+} reduces the proportion of F_v quenched by Q_L from 30 to 16%.

Alkalinization to pH 9.5 was also found to alter the shape of induction kinetics (Fig. 7). A small increase in F_i at pH 9.5 compared to pH 8.0 is seen but no decrease in F_m. The change in sigmoidicity is seen in Fig. 8. For this figure the slow phase of induction has been eliminated by subtraction of an exponential component of appropriate amplitude and rate constant [33]. In Fig. 8A the kinetics of Q reduction (area accumulation) for the remaining fast phase are shown at pH 8.0 and 9.5. At pH 8.0, as observed previously by Melis and Homann [29], a strongly non-linear semi-logarithmic plot is seen illustrating the non-first-order nature of the reaction. At pH 9.5, deviation from first-order kinetics is only slight. This is seen also in Fig. 8B where the F_v vs. area plot diverges only slightly from the straight line obtained for an exponential induction curve. A redox titration of chloroplasts at pH 9.5 reveals a decreased proportion of F_v quenched by Q_L, 22% compared to 30% at pH 8.0 (Fig. 9).

Discussion

In recent years, a series of publications have demonstrated a biphasic redox titration of Q, either by monitoring of room temperature fluorescence under non-actinic illumination [15–17], by observing fluorescence induction in strong light at room temperature [18,23] or at −196°C [20] and by measuring the amplitude of the PS II component of *P*-518 [21]. These results confirm the original observation by Cramer and Butler [14] made in 1969. Two models emerge to explain the existence of the two components, Q_L and Q_H. Firstly, they may be acceptors in the same reaction centre and secondly, they may be acceptors of different PS II units (which may or may not be segregated in the chloroplast membrane system). The purpose of this paper was to see whether observation of the kinetics of fluorescence induction can help to eliminate any of the possible models for the 'Q' heterogeneity; because fluorescence induction is following the course of photoreduction of Q, it is predicted that Q heterogeneity will contribute some features to the induction kinetics.

A model in which Q_L is confined to granal membranes and Q_H to stromal membranes has the attraction of simplicity, and is consistent with the absence of Q_L in 'intermittent light' chloroplasts [19] and with the suggestion that photoreduction of Q_H corresponds to the exponential slow phase of induction [16,25]. However, in a previous paper it has been shown that this slow phase can be eliminated at redox potentials considerably more positive than Q_H [23]. Moreover, it has been shown here that elimination of Q_H leaves an exponential, and not a sigmoidal induction. It should also be stressed that the slow phase only accounts for approx. 12% of the total F_v [30] whereas Q_H accounts for over 50% and most likely 70% [14–20]. Thus, although it may be likely that the slow phase corresponds to a separate pool of Q with E_{m_7} = +120 mV [23], it is clear that the major part of Q_H and Q_L exist in the pigment bed that shows sigmoidal induction. This is shown by the fact that photoreduction of Q_L is faster when Q_H has been reduced than when both Q_L and Q_H are oxidized prior to illumination. If Q_L and Q_H were in separate pigment beds then interaction of this kind would not occur and the induction with Q fully oxidized would be the sum of two independent events (reduction of Q_L and of Q_H).

A correlation between the existence of quenching by Q_L and Q_H and the sigmoidicity of induction is observed in three situations in this paper. Firstly, the potentiometric reduction of Q_H eliminates sigmoidicity when observing photochemical reduction of Q_L. Secondly, Mg^{2+}-depleted membranes show exponential induction kinetics and a 50% decrease in the pro-

portion of Q_L. Thirdly, at pH 9.5, sigmoidicity is decreased along with a 25% decrease in the proportion of Q_L.

Interpretation of the effect of the redox potential on sigmoidicity is complicated. It is predicted that sigmoidicity would decrease when the number of open traps is reduced, since sigmoidicity is proportional to F_v/F_m [5]. Thus, it could be proposed that Q_L and Q_H are randomly distributed in the same pigment bed and that the observed correlation between the elimination of sigmoidicity and Q_H reduction is somewhat coincidental. The correlation between the proportion of Q_L and the sigmoidicity observed after a pH change to 9.5 and after Mg^{2+} depletion would be explained as being due to changes in the organization of the pigment-protein complexes in the thylakoid membranes that alter the quenching properties of Q_L-containing centres.

Thus, a model in which PS II interaction occurs in a domain consisting of reaction centres, the primary electron acceptors of which are either Q_L or Q_H could explain all existing data. The domain would result from aggregation of LHCP-PS II complexes, which of course would be dependent on the ionic environment surrounding the membrane [31]. This scheme would be consistent with the absence of Q_L in membranes lacking the major protein (LHCP) involved in these interactions [19]. It has been proposed earlier that the different E_m values for Q_L and Q_H could, in this scheme, be merely due to redox interaction between two Q's situated close together. Thus, $(QQ) \rightarrow (QQ)^-$ would be the transition described as Q_H and $(QQ)^- \rightarrow (QQ)^{2-}$ as Q_L [19]. Sigmoidicity in this scheme could be due to interaction at the level of either energy transfer or electron transfer.

It has been suggested by Malkin and Barber [17] and Horton and Croze [16] that Q_L and Q_H may be electron acceptors in the same photosystem unit. The simplest model invokes Q_H and Q_L as the transition from Q to Q^- and Q^- to Q^{2-}, respectively [17]. One piece of evidence strongly supporting this view is the observation of strong quenching of fluorescence upon illumination of chloroplasts in the $Q_H^-Q_L^-$ state (but not in the $Q_H^-Q_L$ state) that is thought to result from trapping the reaction centre in the I^- state [17]. If Q_L and Q_H were acceptors in separate systems this quenching would also be expected to result when just Q_H was reduced. In this model, sigmoidal induction would be seen because photochemical reduction of Q_L would be conditional upon Q_H being first reduced. The model implies also that the state $Q_H^-Q_L$ is a less efficient quencher than Q_HQ_L, in agreement with the rise in fluorescence seen when Q_H is reduced potentiometrically. This three-state model was elaborated by Lavorel [12] in 1972 and the mathematical model predicts sigmoidicity only when the relative photochemical rate constants for $[Q_HQ_L \xrightarrow{k_1} Q_H^-Q_L \xrightarrow{k_2} Q_H^-Q_L^-]$ are within certain limits. Changes in ratios of Q_L/Q_H seen in redox titrations and accompanying changes in sigmoidicity can be explained by a decrease in k_2/k_1. Nevertheless, it should also be stated that the two-acceptor and energy-transfer models need not be mutually exclusive. Thus, energy transfer could be the major cause of interaction between PS II units each of which contains Q_H and Q_L as electron acceptors. Because sigmoidicity would depend on quenching efficiency, the above data could be accomodated again if Q_L is a less efficient quencher than Q_H. This scheme resembles that of Joliot and Joliot [33].

A minimal conclusion, therefore, is that Q_L and Q_H are quenchers of fluorescence emanating from the same pigment bed. Although the data are more satisfactorily accomodated in a scheme in which sigmoidicity is explained by a two-photon closure of PS II traps, the generally accepted statistical pigment bed scheme is not excluded. One prediction of the two-photon scheme is that experimental conditions may be found when changes in PS II-PS II interaction can be separated from changes PS II-PS I energy transfer. After divalent cation depletion of membranes, these parameters are closely correlated [32], but in chloroplasts of which the LHCP is phosphorylated, increased energy transfer to PS I is seen without a decrease in PS II-PS II interaction [34].

Acknowledgements

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