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Influence of thylakoid protein phosphorylation on Emerson enhancement and the quantum requirement of Photosystem I

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The relative size of the antennae of PS I and PS II has been estimated from 420 to 722 nm in isolated granal thylakoids, by measuring electron transport from H_2O to NADP upon flash actinic illumination under conditions of linearity of electron transport versus light intensity. Emerson enhancement of NADP reduction by PS I-absorbed radiation of 722 nm had a large peak at 475–500 nm, dropping to zero at 520 nm, and a minor one at 650 nm. No enhancement was observed from 520 to 640 nm, nor at wavelengths above 670 nm. Phosphorylation of LHC-II suppressed the enhancement at all wavelengths: upon phosphorylation, the antenna of PS I became larger than that of PS II over the entire spectrum, including the 475–500 nm region. The quantum requirement of PS I was measured from 420 to 691 nm in the granal thylakoids. A minimum was observed at 520 nm, and maxima at 475 to 500 and 650 to 670 nm, i.e., corresponding to the LHCII maximal absorption.

Introduction

The operation of the light-dependent electron transport system from water to NADP according to the Z scheme requires that the two reaction centres turn over at the same rate. Any departure from this involves the non-photochemical dissipation of the photons absorbed by one photosystem in excess over the other and therefore a decrease in the quantum yield of the process. On the other hand, the two photosystems do not have the same absorption and action spectrum: this is considered the underlying cause of the Emerson 'enhancement' effect [1]. However, the enhancement as discovered by Emerson [2] studying in vivo photosynthesis has been observed in isolated chloroplasts by some authors (Refs. 3,4; see review Ref. 1) but not by others [5,6]. Interestingly, McSwain and Arnon observed enhancement, using 645 nm light as 'light 2' (imbalanced absorption in favor of PS II) and 714 nm as 'light 1' (absorbed mostly by PS I), when CO_2 assimilation by intact chloroplasts was measured, but failed to observe any enhancement when NADP reduction by the same chloroplast preparation was measured after osmotic shock disruption of the chloroplast envelope [6], a treatment which abolishes CO_2 assimilation. It should be noticed that the experiments on enhancement with isolated chloroplasts have been performed in the red region of the spectrum only (usually with light around 650 nm and 700 nm, respectively, as light 2 and light 1), while the maximum enhancement is observed in vivo in the 470–490 nm region and a second, smaller peak around 650 nm [7–9]. Also, previous experiments on enhancement in isolated chloroplasts were performed before the discovery of the adaptation (in vivo) of the photosynthetic apparatus to the prevailing illumination conditions described as the 'state 1-state 2' transitions [10]. The mechanism of such adaptation has been discovered to be dependent upon the phosphorylation of LHC-II and its dephosphorylation, respectively, for state 1 to state 2 and state 2 to state 1 transitions [11–13], and involving the detachment of a fraction of LHC-II from the PS II-LHC-II matrix in the grana partitions. This fraction of LHC-II has been shown to migrate (by lateral diffusion) to the stroma-exposed [14] thylakoids where it becomes associated with PS I antenna [11,13,15]. However, this view is not universally accepted [16].

The activation of LCH-II kinase requires that the intersystem electron carriers are reduced [11,13], and

Abbreviations: LHC-II, light-harvesting chlorophyll *a*/*b* protein complex; PS I, PS II, photosystems I and II, respectively; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; DCIP, 2,-dichlorophenolindophenol; Q_a, the primary quinone acceptor of PS II.

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therefore is dependent upon illumination imbalanced in favor of PS II (or chemical reduction of the intersystem electron carriers, Ref. 17), whilst LHC-II phosphatase seems to have slower activity and to be unregulated [13].

Previous experiments on Emerson enhancement in isolated chloroplasts were performed under conditions where kinase activation state was undefined at the moment of thylakoid preparation, and consequently the state of regulation of the relative size of the PS II and PS I antenna was also undefined.

We have therefore investigated in isolated thylakoids Emerson enhancement, PS I/PS II antennae ratio and the effect of thylakoid protein phosphorylation upon these parameters under conditions in which the thylakoids were in the granal configuration, and LHC-II was initially in the non-phosphorylated state. We have also investigated the quantum yield of PS I under these conditions, from 450 to 690 nm. Our results indicate that enhancement of the H_2O to NADP linear electron transport in isolated, stroma-free stacked thylakoids by 722 nm light is considerably higher in the case of 475–500 nm illumination than of 650 nm illumination. Phosphorylation of LHC-II abolished enhancement, while not affecting the overall electron transport rate, as previously reported [18]. The extent of the PS II antenna decrease upon phosphorylation of LCH-II was the same in the blue and red region (as measured by electron transport or fluorescence decline). The sharp drop of enhancement at 520 nm in the non-phosphorylated thylakoids and the maximum of quantum yield of PS I at this wavelength are suggestive of a relevant contribution of carotenoids to PS I photochemistry.

Methods

Stroma-free thylakoids were prepared as previously described [18] from spinach leaves harvested and kept 2 h at room temperature in darkness. This procedure was adopted to allow dephosphorylation of LHC-II by the endogenous phosphatase [19]. Thylakoids were resuspended in Tricine-NaOH buffer (pH 8) containing 0.4 M sucrose, 10 mM NaCl and 5 mM $MgCl_2$. The same buffer with sucrose 0.1 M served as the reaction medium. Phosphorylation of LHC-II was performed in the dark for 15 min as previously described [18], in the presence of 5 μM ferredoxin, 1 mM NADPH, 1 mM ATP and 10 mM NaF. The control was treated in the same way with the omission of ATP. All samples were then diluted 1 : 50, cooled and stored at 0 °C.

Linear electron transport from H_2O to NADP was measured in the presence of ferredoxin 2 μM and NADP 0.5 mM (PS I + PS II), whilst PS I activity was measured with the further addition of DCMU 5 μM, dithiothreitol 4 mM as the final electron donor and TMPD as the donor to PS I. The same results were obtained when ascorbate 10 mM was substituted for

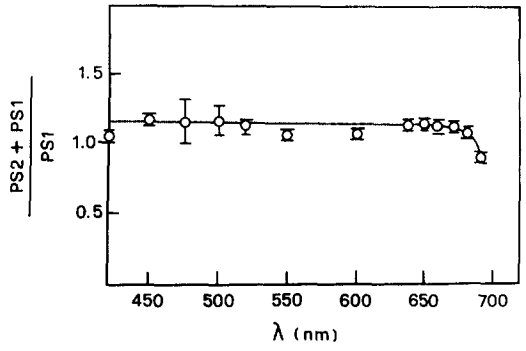

Fig. 1. Ratio of PS I + PS II activity to PS I activity as function of wavelength. Conditions as in Methods. The data were obtained with 26 different preparations, over a period of several months. The bars indicate the standard error.

dithiothreitol. It should be noted that the activity of PS I measured in this way with ferredoxin-NADP as the electron acceptor is underestimated by about 15–20%. Fig. 1 shows indeed that the activity of the H_2O to NADP electron transport requiring PS I + PS II is higher than the one of PS I measured independently in the presence of DCMU and the donor system (Fig. 1) at any wavelength where the activity is limited by PS I antenna size, i.e., up to 690 nm. The cause of this underestimation is probably related to the interaction with ferredoxin of some intermediate formed during the oxidation of the added donor system, as it does not seem to be observed when methylviologen is the electron acceptor (unpublished observations). The system used (DTT or ascorbate as the donor, and TMPD as the lipophylic mediator) was chosen as it gave the highest activity as compared to any other tested.

Chlorophyll concentration (measured according to Arnon, 20) was 15 μg/ml. Actinic illumination was always checked to be low enough to give a reaction velocity strictly proportional to energy flux. Under these conditions, no effect of the addition of uncouplers or ADP plus P_i was observed. NADP reduction was measured as previously reported [15], in a dual wavelength spectrophotometer (Sigma, Munich) as the 340 minus 390 nm absorbance change. Side-illumination with actinic light was provided at 90° with respect to the measuring beam. The photomultiplier was protected from the actinic beam and fluorescence emission by a Corning 7-60 filter and a saturated copper sulfate solution of 1 cm light path. Actinic illumination was provided by xenon flashes of 3 to 10 μs duration (90% of the energy was delivered in 10 μs) at the frequency of 6/s, filtered through a Calflex heat filter (absorbing all radiation above 720 nm) and interference filters of 8 to 10 nm halfband width, as indicated. Light intensity was attenuated by means of neutral filters. Under these conditions, the measured electron transport is a function of the efficiency of light harvesting and of energy transfer to the reaction centers whilst it is independent

of the rates of secondary electron transport reactions. Therefore, under these conditions the estimation of electron transport as a function of wavelength is a measure of the antenna size at any wavelength. When required for Emerson enhancement experiments, the sample cuvette was illuminated from both sides with the actinic beams, as indicated. In these experiments light absorbed mostly by PS I, when given, was a continuous beam of 722 nm of intensity high enough to ensure that all PS II centres were open when the lower wavelength flashes were fired at the rate of 6 per s (see Fig. 2).

The intensity of actinic light transmitted by each filter was measured by means of a radiometer (model 65, YSI, Ohio) calibrated with a chemical actinometer [21] in the cell compartment of the spectrophotometer. In the case of quantum yield measurements, the absorption spectrum of the thylakoid suspensions was measured in a double-beam spectrophotometer connected to a computer, and light scattering was corrected for by the opal glass method of Shibata [22]. Absorbance was evaluated according to Amesz et al. [23], and converted to $1 - T$ values (absorptance). The energy of incident radiation transmitted by each interference filter was calculated from the absorption spectrum of the filters recorded in the same instrument and the measured incident energy. These data served to calculate the absorbed energy.

Fluorescence emission was measured at 690 nm and excited through a Corning 4-96 filter. The fluorimeter cuvette contained the thylakoid suspension (5 μg of chlorophyll/ml) and DCMU 10 μM.

Results

The PS II + PS I-dependent NADP reduction is shown in Fig. 2A (650 nm actinic light as λ_2) without and with the addition of non-modulated 722 nm light (as λ_1) supplied at two intensity values, showing that the lower one was high enough to saturate the enhancement effect. The PS-I-dependent reaction in the presence of DCMU and the electron donor system is shown in Fig. 2B. The maximal activity of PS II at any particular wavelength, λ_2, can be calculated as the difference between the reaction velocity measured when both wavelengths are supplied, $V(\lambda_{2+722})$, and that measured with 722 nm light only (V_{722}), i.e., $V(\text{PSII}) = V(\lambda_{2+722}) - V_{722}$, provided the 722 nm energy is high enough to give the maximal value of the above difference, as was the case in our experiments, at the intensity of the actinic λ_2 beam used (see Fig. 2A). The Emerson enhancement, E_2, (see Ref. 1), can be calculated as $[V(\lambda_{2+722}) - V_{722}]/V(\lambda_2)$. Under the conditions adopted therefore $Q_a(\lambda_{2+722}) = Q_a(722)$, i.e., the concentration of oxidized Q_a under illumination with λ_{2+722} nm light is approximately equal to that observed under illumination with 722 nm light only, and $E_2 = E_{2\max} = Q_{a1}/Q_{a2}$, where Q_{a1} and Q_{a2} indicate, respectively, the fraction of Q_a in the oxidized state upon illumination with light 1 (of 722 nm) and light 2 (see Ref. 1). Under these conditions, E_2 is indicative of the ratio of the antennae of PS II to that of PS I.

The maximum of E_2 was observed at 475 nm and 500 nm, a spectral region where most of the absorption

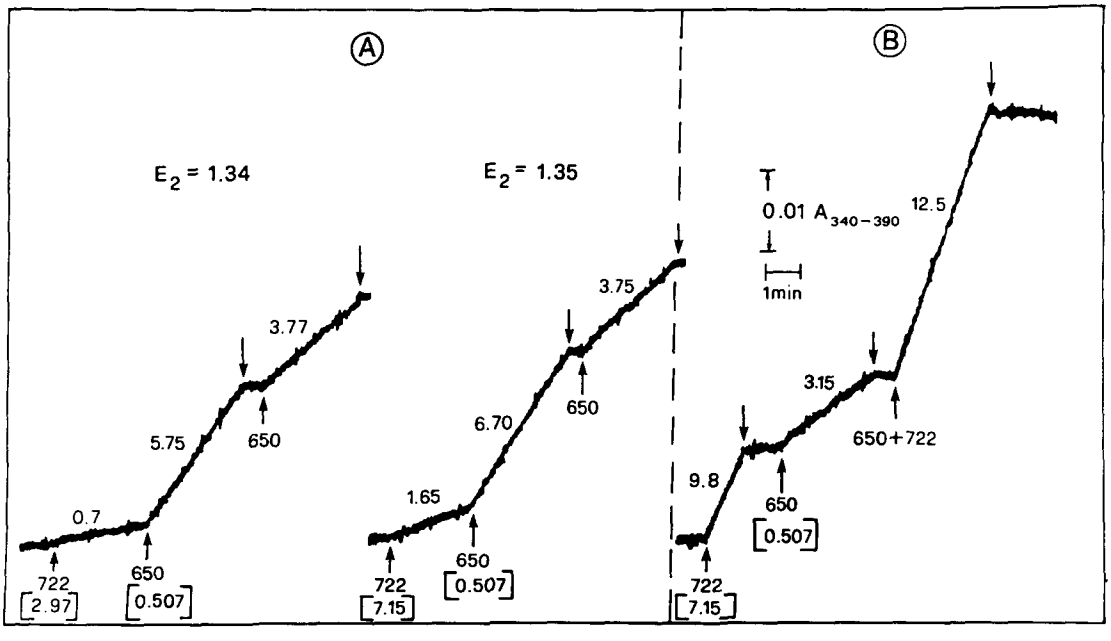

Fig. 2. Spectrophotometer recorder trace of NADP reduction in the PS II + Ps I- and PS-I-dependent system. Conditions as in Methods. (A) PS II + PS I electron transport from H_2O to NADP. (B) PS-I-dependent electron transport in the presence of the donor system. Upward arrows: light one; downward arrows: light off. The wavelength of actinic light is indicated below the arrow in nm and the energy (in brakets) in $W \cdot m^{-2}$. The figures above the traces indicate the activities, $\Delta A_{340-390}$/min.

is due to chlorophyll *b* (and therefore to LHC-II) and to the carotenoids. At 520 nm a sharp decrease of enhancement down to 1.03 was observed. The enhancement was very small all over the green and red part of the spectrum, except for a small peak at 650 nm, very close to the value of 1.16 reported by Joliot et al. [4], who measured O_2 evolution coupled to NADP reduction by means of the rate electrode.

The phosphorylation of LHC-II (and of other thylakoid proteins) has been claimed to be the biochemical mechanism causing the state 1–state 2 transition [11–13], i.e., the increase of PS I and decrease of PS II antenna. Phosphorylation therefore should decrease the enhancement of NADP reduction rate by 722 nm light under our conditions. Indeed, enhancement was abolished and the PS I/PS II ratio was raised above unity at all wavelengths from 475 to 650 nm by the phosphorylation of the thylakoid proteins (Table I). No effect was observed at 722 nm, as previously reported [15]. The decrease of PS II activity upon phosphorylation was very similar at all wavelengths (about 20%), and corresponded closely to the fluorescence decrease (Table I).

The quantum requirement of PS I photochemistry was measured in grana-stacked, non phosphorylated thylakoids from 450 to 691 nm (Fig. 4). The quantum requirements reported should be considered about 15–20% overestimated, due to the underestimation of the PS I activity (see Methods). However, this does not affect the relative quantum requirement as a function of wavelength. Illumination was supplied as short flashes

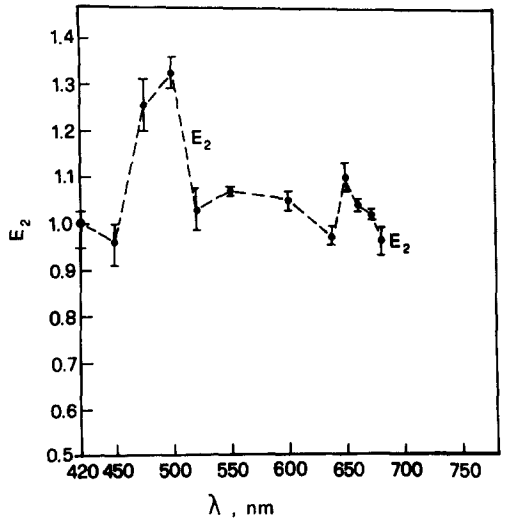

Fig. 3. Emerson enhancement (E_2) as function of wavelength. Conditions as in Methods. The data were obtained, in duplicate samples, from 26 different thylakoid preparations from 26 spinach cultures. In each experiment 3 or 4 wavelengths were tested. The bars indicate the standard error. PS II was calculated as $V(\lambda_{2+722}) - V_{722}$, and E_2 as $[(V(\lambda_{2+722})) - V_{722}]/V(\lambda_2)$.

TABLE I

Effect of thylakoid protein phosphorylation on PS II and PS I antennae and on enhancement

Conditions: as in Methods. NP, non phosphorylated and, P, phosphorylated thylakoids. The figures indicate NADPH formation as $10^\circ \times \Delta A_{340-390} \cdot \text{min}^{-1}$, ±S.E. PS I was measured in the presence of the donor system and DCMU (see Methods). PS I + PS II is the electron transport from H_2O to NADP at the indicated wavelength (Vλ), PS II = $(V(\lambda + _{722}) - V_{722}$, and $E_2 = [V(_{\lambda + 722}) - V_{722}]/V_\lambda$ (see text). Fluorescence decrease upon phosphorylation was 22.8% ± 4.7.

λ (nm)		PS I	PS I + PS II	PS II	E_2	PS I/PS II
475						
(0.774 $W \cdot m^{-2}$)	N.P.	5.03 ± 0.7	6.04 ± 0.73	7.42 ± 0.81	1.23 ± 0.13	0.68
	P	6.57 ± 1.04	6.15 ± 0.74	5.86 ± 0.53	0.95 ± 0.04	1.12
	P/N.P.	1.30	1.02	0.79	–	
500						
(0.516 $W \cdot m^{-2}$)	N.P.	4.59 ± 0.30	5.52 ± 0.28	6.86 ± 0.14	1.24 ± 0.04	0.67 ± 0.03
	P	6.09 ± 0.34	5.09 ± 0.18	5.12 ± 0.20	0.99 ± 0.03	1.18 ± 0.03
	P/N.P.	1.33	0.92	0.75	–	
520						
(0.968 $W \cdot m^{-2}$)	N.P.	4.50 ± 0.45	5.19 ± 0.54	5.63 ± 0.89	1.00 ± 0.10	0.81 ± 0.1
	P	5.62 ± 0.83	4.91 ± 0.43	4.66 ± 0.39	0.97 ± 0.08	1.2 ± 0.09
	P/N.P.	1.25	0.96	0.83	–	
650						
(0.417 $W \cdot m^{-2}$)	N.P.	3.88 ± 0.15	4.38 ± 0.39	4.86 ± 0.69	1.13 ± 0.04	0.80 ± 0.10
	P	4.39 ± 0.39	4.02 ± 0.61	3.86 ± 0.28	0.93 ± 0.08	1.13 ± 0.07
	P/N.P.	1.13	0.92	0.79	–	
722						
(2.97 $W \cdot m^{-2}$)	N.P.	4.77 ± 0.63	0.61 ± 0.17	0.61 ± 0.17	–	7.82
	P	4.48 ± 0.71	0.62 ± 0.15	0.62 ± 0.15	–	7.22
	P/N.P.	0.94	1.02	1.02	–	

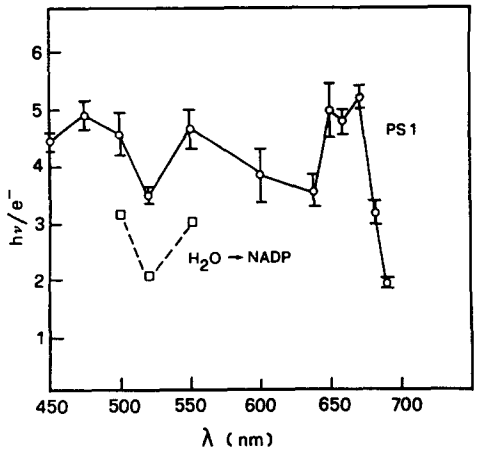

Fig. 4. Quantum requirement of PS I. Conditions as under Methods. The bars indicate the standard error. The data are the average of several preparations of thylakoids, over a period of several months.

of 3 to 10 μs duration at the frequency of 6 per s. This frequency is adequate to allow the complete relaxation of the system in the dark period between two flashes, as proven by the observation that the ratio electron transport/flash was unchanged when the frequency was halved (not shown). Light intensity was low enough to ensure linearity of the reaction rate versus energy flux. When the measurements were performed at higher light intensity (off the linearity of rate versus intensity) and the data were plotted as $h\nu$/rate versus light intensity, lower quantum requirements were found by extrapolation to zero intensity (not shown), as previously reported [26]. A minimum quantum requirement of PS I was observed at 520 nm (Fig. 4), a wavelength at which the carotenoids contribute more than the chlorophylls to absorption, whilst a maximum requirement was observed at 650 to 670 nm, and dropped sharply above this wavelength. The quantum requirement of the H_2O to NADP system also had a minimum at 520 nm (Fig. 4), and was lower than the PS I requirement. This was expected in the presence of Mg ions, a condition where the thylakoids are granal and the spillover of energy from the PS II-LHC-II matrix to PS I is prevented, so that no energy absorbed by PS II can be utilized by PS I.

Discussion

Our observations on the size of the antenna of PS I and PS II in isolated thylakoids kept in the granal state and on the Emerson enhancement (as far as we know, this is the first report of enhancement in the blue region of the spectrum in isolated thylakoids) are consistent with the widely accepted idea [1,7,8] that the PS II antenna is larger than that of PS I along most of the spectrum, except at wavelengths above 680 nm. We show here that the imbalance in favor of PS II is particularly large at 475 and 500 nm, corresponding to the peak of chlorophyll *b* and therefore of LHC-II absorption, and to carotenoid absorption. Correspondingly, at these wavelengths we observed the maximum enhancement of electron transport upon addition of PSI-absorbed light of 722 nm. The sharp increase of PSI antenna size and quantum yield at 520 nm reported here (Figs. 3 and 4) is in agreement with the in vivo observations of Canaani and Malkin [8], who found a minimum of the PS II/PS I antennae ratio at this wavelength. Garlaschi et al. [27] measured the steady-state level of oxidised Q_a in thylakoid preparations similar to ours under conditions of constant PS II absorption. They reported a large minimum around 480 nm and a smaller one at 650 nm, in agreement with our spectrum of E_2.

It is possible that the failure of some authors [6] to observe enhancement in isolated chloroplasts reducing NADP is due to the fact that their observations were limited to the red region of the spectrum, where enhancement is rather small (see Fig. 3 and Ref. 4). This is a major difference with the situation in vivo, where enhancement is readily observed with a large peak around 475 nm, but also another from 550 to 600 nm and again in the red with the maximum around 650 nm [7,8]. It seems likely that the larger enhancement observed in vivo (or, for the same reason, in isolated intact chloroplasts [6] reducing CO_2) is the consequence of the requirement for additional PSI activity to drive cyclic electron transport coupled to ATP synthesis, more than to implement PS I activity of the linear electron transport to NADP. It may be relevant in this respect to note that McSwain and Arnon [6] observed enhancement at 650 nm in intact chloroplasts assimilating CO_2, but not in the same preparations reducing NADP after osmotic shock: in the former case ATP synthesis was required for the operation of the pentose phosphate cycle and therefore for continued electron transport, not in the latter where substrate amount of NADP were available. More recently, Horton observed in isolated intact chloroplasts that the higher the requirement for ATP, the larger is the enhancement [28]. Sinclair and Cousineau reported enhancement by 700 nm light of NADP reduction by thylakoids illuminated with 640 nm modulated light [29], under conditions similar to ours. They also observed that the addition of ATP caused the simultaneous decrease of enhancement and of PS II fluorescence, while not affecting the rate of O_2 evolution.

We have shown (Table I) that the phosphorylation of LHC-II abolishes the enhancement by 722 nm light of electron transport to NADP at any wavelength, while increasing the PS I antenna size and decreasing the antenna of PS II of the same amount as that evaluated on the basis of fluorescence decrease (Table I). While the decrease of PS II antenna upon phosphorylation of LHC-2 and other thylakoid proteins is generally

accepted [11,13], the increase of PS I antenna [11–15] has been challenged [16,25]. The effect of protein phosphorylation on enhancement, reported here (see also Ref. 29) is a further argument in favor of the idea that phosphorylation increases PS I antenna, based on a different experimental method where no artificial electron donor to PS I is used, and steady-state electron transport is measured under conditions where the size of the antennae is evaluated. If phosphorylation of LHC-II were simply to decrease the efficient PS II alpha centres as claimed by Melis and collaborators [16,25], phosphorylation would suppress or decrease enhancement but would also decrease the overall NADP reduction, which was not observed (see Table I).

The quantum requirement of PS I shown in Fig. 4, measured under conditions where it reflects the size and efficiency of the antenna, is consistent with the enhancement (E_2) values reported in Fig. 3. The sharp drop of E_2 at 520 nm coincides with the peak of PS I efficiency at this wavelength, also observed in vivo [8]. It is possible that the carotenoids connected with PS I are responsible for this high efficiency at 520 nm.

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