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Protein tyrosine phosphorylation in the transition to light state 2 of chloroplast thylakoids

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Abstract

Redox dependent protein phosphorylation in chloroplast thylakoids regulates distribution of excitation energy between the two photosystems of photosynthesis, PS I and PS II. Several thylakoid phosphoproteins are known to be phosphorylated on N-terminal threonine residues exposed to the chloroplast stroma. Phosphorylation of light harvesting complex II (LHC II) on Thr-6 is thought to account for redistribution of light energy from PS II to PS I during the transition to light state 2. Here, we present evidence that a protein tyrosine kinase activity is required for the transition to light state 2. With an immunological approach using antibodies directed specifically towards either phospho-tyrosine or phospho-threonine, we observed that LHC II became phosphorylated on both tyrosine and threonine residues. The specific protein tyrosine kinase inhibitor genistein, at concentrations causing no direct effect on threonine kinase activity, was found to prevent tyrosine phosphorylation of LHC II, the transition to light state 2, and associated threonine phosphorylation of LHC II. Possible reasons for an involvement of tyrosine phosphorylation in light state transitions are proposed and discussed.

Abbreviations: ATP – adenosine triphosphate; BSA – bovine serum albumin; DMSO – dimethylsulphoxide; DTT – 1,4-dithiothreitiol; LHC II – light harvesting complex II; PS I – Photosystem I; – PS II, Photosystem II; SDS–PAGE – sodium dodecylsulphate polyacrylamide gel electrophoresis

Introduction

Post translational modification of proteins by reversible phosphorylation regulates protein structure and function (Johnson and Barford 1993). Of the enzymes responsible for phosphotransfer, the best characterised are the serine/threonine kinases and the tyrosine kinases (Hunter and Cooper 1985; Edelman et al. 1987) which catalyse phosphorylation on serine/threonine residues and tyrosine residues, respectively. Protein phosphorylation events are directly involved in controlling many cellular events in plants and other eukaryotic organisms as well as in prokaryotes.

The light-harvesting complex of PS II of chloroplast thylakoids, LHC II, is an oligomeric, intrinsic membrane protein that binds chlorophylls and carotenoids (Thornber 1975). The function of LHC II is to collect light energy for transfer to reaction centres of the two photosystems of photosynthesis in chloroplasts. Certain LHC II polypeptides are phosphorylated on threonine residues. These threonine residues are located near the amino-terminus and exposed at the outer, stromal surface of the thylakoid (Bennett 1979). The functional effect of this phosphorylation is to decrease the affinity of the LHC II for PS II and increase its affinity for PS I. Phosphorylation thereby redistributes absorbed light energy to PS I at the expense of PS II, and provides a molecular basis for the transition to light state 2, a state of adaptation to illumination favouring PS II (Allen et al. 1981). The complementary transition, to light state 1,

involves dephosphorylation of LHC II and occurs in response to illumination favouring PS I. The kinetics of chloroplast thylakoid protein phosphorylation and dephosphorylation match the kinetics of the state 2 and state 1 transitions, respectively (Telfer et al. 1983). Many independent studies support a role for LHC II phosphorylation in state transitions in plants and green algae (Allen 1992).

Cyanobacteria are prokaryotes related to the chloroplast. Light-dependent tyrosine kinase activity has been reported in the chlorophyll-*b*-containing cyanobacterium *Prochlorothrix hollandica* (Warner and Bullerjahn 1994). Even though no tyrosine kinase has been isolated and characterised in higher plants there are several reports of plant protein tyrosine kinase activities (Hirayama and Oka 1992; Trojanek et al. 1996; Xu et al. 1998; Barizza et al. 1999). Reversible protein tyrosine phosphorylation has been found to be involved in plants' responses to environmental stimuli (Xu et al. 1998). By use of anti-phosphotyrosine antibodies, acid and alkali treatment, and specific chemical inhibition, LHC II and other chloroplast thylakoid membrane proteins have been shown to be phosphorylated on tyrosine as well as threonine (Tullberg et al. 1998). Does this protein tyrosine kinase activity play any part in the state 2 transition, or is phosphorylation on threonine sufficient to explain this regulatory phenomenon? Here, we describe results showing that changes in thylakoid protein tyrosine phosphorylation accompany changes in chlorophyll fluorescence emission induced by illumination specific to either PS I or PS II. In addition, the tyrosine kinase inhibitor genistein has concentration-dependent, specific effects on both tyrosine phosphorylation and state transitions. We suggest that protein phosphorylation on tyrosine is involved in the transition to light state 2. We discuss the requirement for both threonine and tyrosine phosphorylation in regulation of light-harvesting function in chloroplast thylakoids.

Materials and methods

Plant material and isolation of thylakoids

Peas (*Pisum sativum* L. cv Oregon sugar pod) were grown on vermiculite in a 20 °C growth chamber with a 12 h dark/12 h light cycle (light intensity of 25 μE m^{-2} s^{-1}). Thylakoids were isolated from 12-to-15-day-old pea leaves (Walker 1971). Chlorophyll concentrations were estimated by the method of Arnon (1949).

In vitro *phosphorylation – time course and genistein titration*

Time course. A suspension of isolated thylakoids at a chlorophyll concentration of 0.2 mg/ml, in a medium containing 0.1 M sorbitol, 50 mM HEPES pH 7.6, 5 mM of MgCl, NaCl and NH_4Cl (phosphorylation medium) was divided in 100 μl aliquots. The thylakoids were dephosphorylated on ice in darkness for at least 1 h. Prior to phosphorylation, and under a low intensity green safe light, NaF was added to a final concentration of 10 mM to the dark-adapted thylakoids. The phosphorylation reaction was initiated by addition of ATP to a final concentration of 200 μm. The reaction was terminated by addition of an equal volume of twice-concentrated electrophoresis buffer (70 mM Tris (pH 6.8), 4.4% SDS, 100 mM DTT and 12.5% glycerol) after 2, 5, 10 and 25 min of incubation at 26 °C and a light intensity of 80 μE m^{-2} s^{-1}. Illumination was provided by fluorescent strip lights (3× 18 W). Dark-adapted thylakoids without addition of ATP were considered as time zero in the time course.

Genistein titration. The tyrosine kinase inhibitor genistein (4′,5,7-trihydroxyisoflavone, Sigma-Aldrich Corporation, Sigma cat. no. G6649) was dissolved in DMSO. Thylakoids suspended in phosphorylation medium at a chlorophyll concentration of 0.2 mg/ml were dark-adapted as above. Dark-adapted thylakoids were incubated in the presence of 0 (DMSO only), 5, 15, 35 and 75 μm genistein for 5 min before addition of NaF. The phosphorylation assay was then continued as described above. The reaction was allowed to proceed for 10 min. DMSO concentration was 1% (v/v) in all samples containing genistein and in the DMSO-control.

SDS–PAGE and immunoblotting

Phosphorylated samples were heated to 70 °C for 7 min in electrophoresis buffer (35 mM Tris (pH 6.8), 2.2% SDS, 50 mM DTT and 6.25% glycerol). Thylakoid proteins were separated by SDS–PAGE (14% w/v acrylamide of which 2.6% was cross-linker) essentially as described by Laemmli (1970) using the mini-gel system of BioRad, following the manufacturer's instructions.

The proteins were transferred from the gels (200 mA; 30 min) to nitrocellulose membrane (Hy-

bond ECL, Amersham-Pharmacia Biotech) by semi-dry electroblotting in a horizontal unit (Multiphore II/NovaBlot, Pharmacia). The transfer buffer was as described by Bjerrum and Schafer Nielsen (1986). After transfer, the membranes were washed in TBS (20 mM Tris (pH 7.6), 140 mM NaCl) and blocked in TBS containing 3% BSA (Fraction V, 98% pure, Sigma-Aldrich Corporation) either over night at 4 °C or for several hours at room temperature. Phosphoproteins were immunodetected using anti-phospho-tyrosine and anti-phospho-threonine antibodies (Zymed Laboratories Inc.) and a chemiluminescent protein detection system (Immun-Star™, BioRad). Quantification of immunoreactions was carried out with a Personal Densitometer SI (Molecular Dynamics) and the software ImageQuant (Molecular Dynamics).

Modulated chlorophyll fluorescence measurements

Modulated chlorophyll fluorescence measurements were made using a Walz PAM Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Dark-adapted thylakoids were illuminated with PS II-enriched light (PAM 101 light emitting diode, 650 nm) for 5 min, during which time fluorescence emission reached a steady state. ATP (to a final concentration of 200 μm) was then added. After 5 min of further measurements, PS I-enriched light (PAM 102 FR light emitting diode, 730 nm) was added to the PS II light. The measurements were then continued for another 6 min before the PS I light was turned off again. Chlorophyll fluorescence was then monitored for another 4 min. At low light intensities of PS II light, PQ redution is reversible even in the absence of an added electron acceptor, and turning off the light initiates protein dephosphorylation and the fluorescence rise indicating a transition to state 1 (Bennett et al. 1980; Silverstein et al. 1993).

Samples containing genistein were incubated in the presence of the genistein (at final concentrations 5, 15 or 35 μm) for 5 min prior to the start of the measurements. Control samples were preincubated in the same way with the solvent DMSO. DMSO concentration in all genistein-containing samples and in the DMSO-controls was 1% (v/v). In all measurements, the thylakoids were suspended in phosphorylation medium at the same chlorophyll concentrations as the *in vitro* phosphorylation, in a stirred glass cuvette, maintained at 20 °C. The experiments were carried out under a low intensity green safety light.

Low temperature fluorescence emission spectroscopy

77 K fluorescence emission spectra were recorded for phosphorylated and non-phosphorylated thylakoids and for thylakoids preincubated with genistein (35 μm) before phosphorylation. After phosphorylation or dephosphorylation the samples were diluted 15-fold in phosphorylation medium containing 30% (v/v) glycerol and then rapidly frozen to 77 K. With fluorescence excitation set at 440 nm, fluorescence emission was measured continuously between 600 and 800 nm using a Perkin-Elmer LS-5 Luminescence Spectrometer. Slit width of the excitation beam was 10 nm and the slith width for emission was 5 nm. The phosphorylation and dephosphorylation procedures were carried out essentially as described above. All spectra were normalized to the major peak around 735 nm. Spectra were digitised and stored using a MacLab A/D converter with associated software (Chart v3.3.5).

Results

In vitro *phosphorylation and immunoblotting*

Phosphorylation of several thylakoid proteins was revealed through the use of specific antibodies directed towards phospho-threonine and phospho-tyrosine. In agreement with previous reports, reviewed in Allen (1992), known thylakoid phosphoproteins, for example D1 and D2, were found to be phosphorylated on threonine residues (results not shown). LHC II was found to be phosphorylated on tyrosine and on threonine residues. The phosphorylation of both tyrosine and threonine residues on LHC II was found to increase in a time-dependent manner, with an apparent $t_{1/2}$ of about 4 min (Figures 1a, b).

Increasing concentrations of the tyrosine kinase inhibitor genistein could be seen to be correlated with decreasing extents of phosphorylation of LHC II on both tyrosine and on threonine residues (Figures 1c, d). The concentration of genistein that produced 50% inhibition of LHC II-tyrosine phosphorylation (IC_{50}) was found to be approximately 15 μm. Genistein at 15 μm final concentration was also found to give about 50% inhibition of LHC II-threonine phosphorylation (Figures 1c, d), indicating that the genistein concentration-dependency of the inhibition of LHC II tyrosine phosphorylation is similar to that of LHC II threonine phosphorylation. The phosphorylation of thylakoid proteins other than LHC II was found to be sensitive to genistein (results not

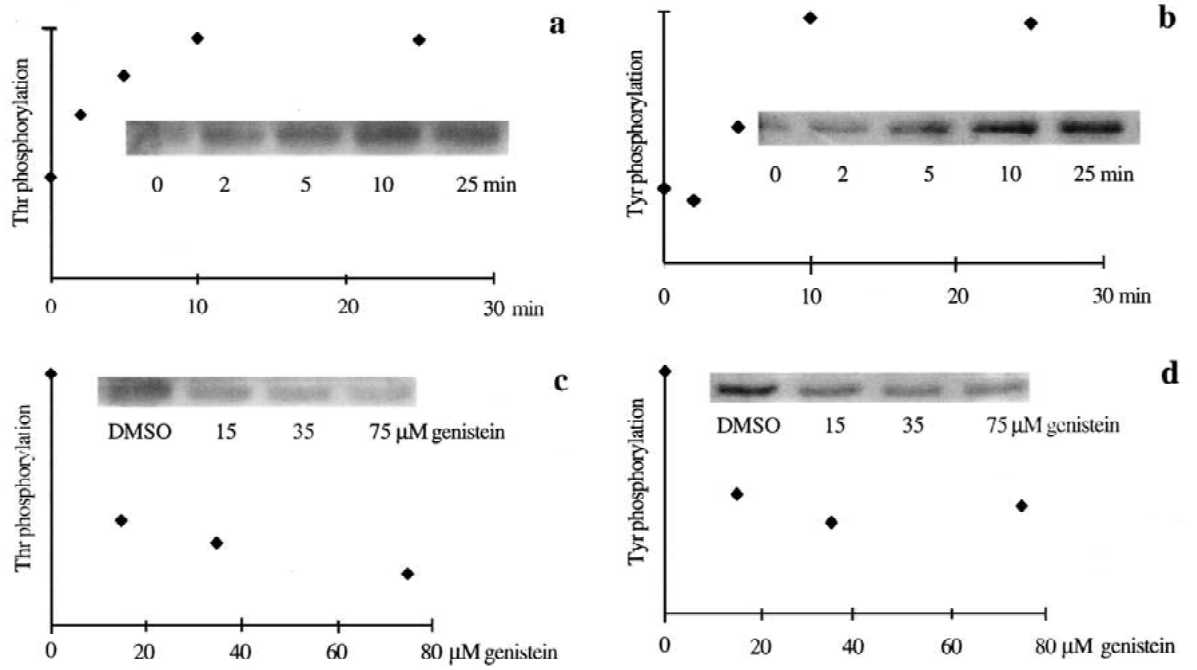

Figure 1. Immunoblots of LHC II phosphorylation and quantification of the signal. (a) time course of phosphorylation on LHC II threonine residues. (b) time course of phosphorylation on LHC II tyrosine residues. (c) effect of genistein at different concentrations (0–75 μM) on phosphorylation of LHC II threonine residues. (d) effect of genistein at different concentrations (0–75 μM) on phosphorylation of LHC II tyrosine residues.

shown) as reported also by Tullberg et al. (1998). D1/D2-phosphorylation was also found to decrease with increasing concentrations of genistein (results not shown).

Modulated chlorophyll fluorescence measurements

Isolated thylakoids, illuminated with PS II enriched light, showed a quenching of chlorophyll fluorescence of approximately 15% over a time period of 5 min (Figure 2a) following the addition of ATP. This fluorescence decrease is the result of redistribution of excitation energy from PS II to PS I. Redistribution of excitation energy to PS I is described as the transition to state 2, and is dependent on phosphorylation of LHC II. Phosphorylation of LHC II causes movement of phospho-LHC II away from PS II to PS I where phospho-LHC II acts as an antenna, supplying excitation energy to PS I (Allen et al. 1981; Telfer et al. 1983). The decrease in fluorescence was still present to the same degree after preincubation of the thylakoids in the presence of 1% (v/v) DMSO and addition of ATP (Figure 2b). Conversely, preincubation of thylakoids with the protein tyrosine kinase inhibitor genistein led to inhibition of the quenching of chlorophyll fluorescence when ATP was added (Figures 2d, e). The inhibition of the transition to light state 2 is seen to be dependent on genistein concentration (Figures 2d, e) with an IC_{50} of 13 μm. The presence of the uncoupler gramicidin had no effect on the pattern of results seen in Figure 2 (not shown).

Addition of PS I light rapidly decreased chlorophyll fluorescence emission by 20% in all samples (Figure 2), as PS II traps for excitation are opened, that is, as photochemical quenching of PS II fluorescence is increased. Fluorescence then increased. This gradual increase of chlorophyll fluorescence upon PS I light addition, the transition to state 1, indicates a redistribution of excitation energy towards PS II (Allen 1992). This slow, rising phase was decreased in, or absent from, samples which had been preincubated with genistein (Figures 2d, f) or when NaF was present (Figure 2c). The fluorescence increase is a result of dephosphorylation of phospho-LHC II associated with PS I and the subsequent migration of LHC II back to PS II (Allen et al. 1981; Telfer et al. 1983). NaF inhibits the dephosphorylation of thylakoid phosphoproteins including phospho-LHC II (Bennett 1980) and therefore inhibits the transition to light state 1 (Telfer et al. 1983). In contrast to the inhibitory effect of NaF

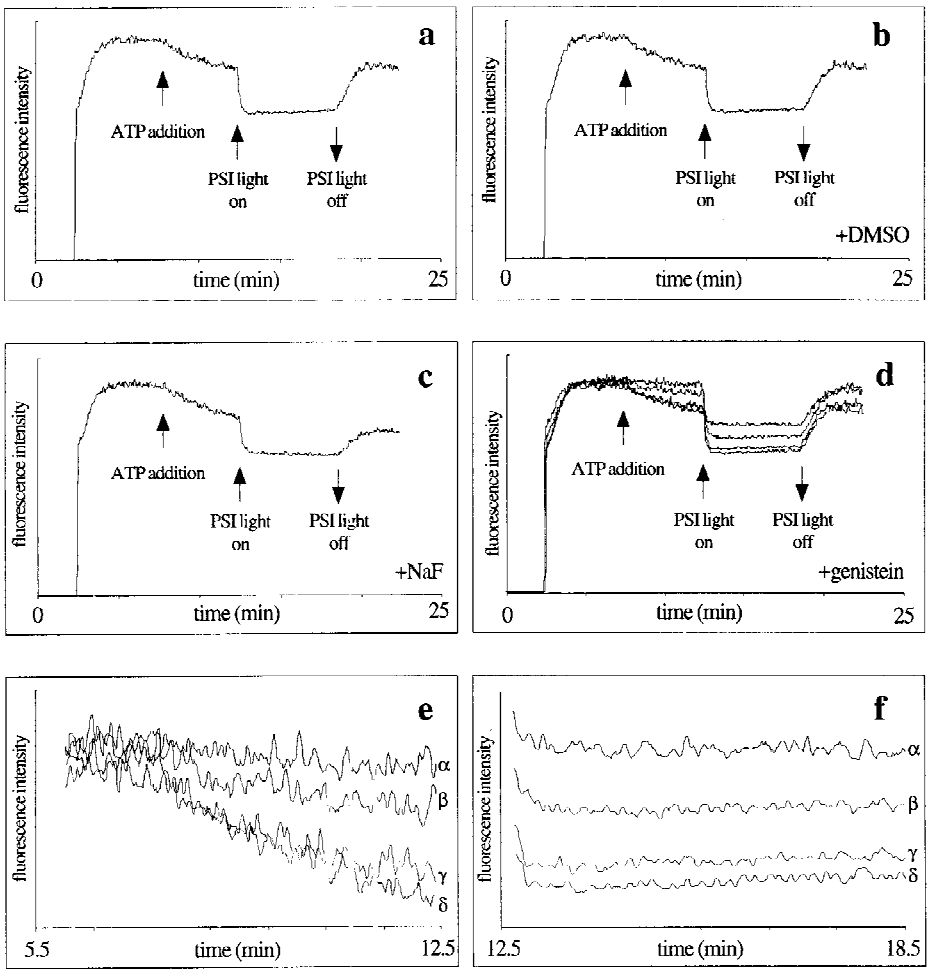

Figure 2. Modulated chlorophyll fluorescence measurements of thylakoids. Fluorescence was excited by modulated PS II light. Fluorescence of thylakoids (a) with no extra addition prior to start of the measurements; (b) in the presence of 1% DMSO; (c) in the presence of 10 mM NaF; (d) in the presence of genistein at different concentrations (see e for details). (e) Enlargement of panel d between 5.5 min and 12.4 min (the transition to light state 2), α: 35 μM genistein, β: 15 μM genistein, γ: 5 μM genistein, δ: 0 μM genistein (DMSO-control). (f) Enlargement of panel d between 12.8 min and 18.5 min (transition to light state 1). The traces are described as in (e).

on the transition to state 1, genistein inhibits the preceding state transition, the transition to state 2, which depends on LHC II phosphorylation and the migration of phospho-LHC II towards PS I. Consequently, the dephosphorylation effect upon addition of PS I light, was decreased or absent when genistein was present (Figures 2d, f).

Low temperature fluorescence spectroscopy

At 77 K, fluorescence emission in the wavelength interval 600–800 nm from thylakoids excited by light with wavelength of 440 nm reveals two major emission peaks. The peaks are at approximately 685 nm and 735 nm, respectively (Figure 3). Fluorescence around 685 nm originates from PS II whereas PS I has a fluorescence emission maximum at 735 nm. In non-phosphorylated thylakoids, the PS II fluorescence emission (at 685 nm) was 90% of the PS I fluorescence emission (at 735 nm) and in phosphorylated thylakoids the 685 nm peak was 65% of the 735 nm peak, judged by the heights of the peaks. For phosphorylated thylakoids that had been treated with genistein at 35 μm final concentration, the 685 nm peak height was 75% of the 735 nm peak height (Table 1). Integration reveals that total PS II fluorescence emission was reduced about 35% after phosphorylation of the thylakoids compared to the non-phosphorylated thylakoids, while total PS II fluorescence emission

Table 1. Fluorescence emission values from the low temperature spectra given in Figure 3, of non-phosphorylated thylakoids (control), phosphorylated thylakoids (+ATP) and thylakoids phosphorylated in the presence of genistein (+ATP, genistein). The values in the columns 'Height' are based on the amplitude of fluorescence emission at the two maxima, at 685 nm and 735 nm. The values in the columns 'Area' are based on the value for the total area bounded by the corresponding band in the fluorescence emission spectrum. Fluorescence emission is measured directly in arbitrary units: spectra were normalised to F_{735}

	F_{685}		F_{735}		F_{685}/F_{735}		$F_{685}/F_{685-control}$	
	Height	Area	Height	Area	Height	Area	Height	Area
Control	0.90	9.96	1.00	21.13	0.90	0.47	1.00	1.00
+ATP	0.64	6.43	0.99	21.84	0.65	0.29	0.71	0.65
+ATP, genistein	0.76	7.63	1.01	21.22	0.75	0.36	0.84	0.77

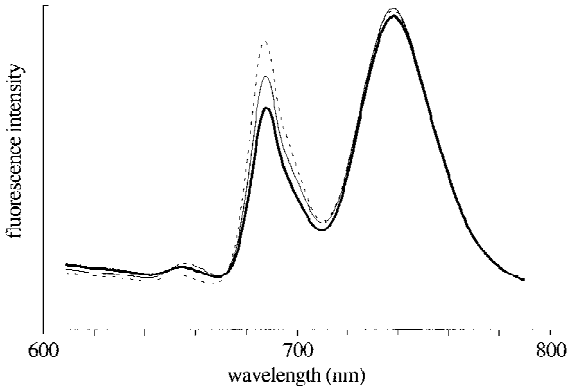

Figure 3. Low temperature fluorescence emission spectra for non-phosphorylated thylakoids (dashed line), phosphorylated thylakoids (broad line) and for thylakoids preincubated with genistein (35 μM) before phosphorylation (narrow line). The fluorescence excitation wavelength was 440 nm.

from phosphorylated, genistein-treated thylakoids was reduced by only 23% (Table 1). The relative decrease in PS II fluorescence emission results from phosphorylation of LHC II and its subsequent detachment from PS II reaction centers and migration to PS I reaction centers, the transition to light state 2. Samples which had been pre-incubated with genistein showed a smaller reduction in PS II fluorescence emission upon phosphorylation, and are therefore inhibited in the transition to light state 2.

Discussion

Phosphorylation of Thr-6 of LHC II (Mullet 1983; Michel et al. 1988) is thought to induce structural changes in the N-terminus of LHC II, break up the LHC II trimers and allow migration of monomeric phospho-LHC II from PS II towards PS I (Nilsson et al. 1997). Once phospho-LHC II becomes associated with PS I, it acts as an antenna complex feeding excitation energy into PS I (Allen et al. 1981). Our results imply that this event also requires tyrosine kinase activity in addition to the threonine phosphorylation. If tyrosine phosphorylation is required in this process, it is expected that the light state 2 transition is sensitive to the tyrosine kinase inhibitor genistein. By using modulated fluorescence (Figure 2) and low temperature fluorescence spectroscopy (Figure 3) to monitor the occurrence of state transitions in the absence and presence of genistein we observed that the light state 2 transitions are indeed inhibited in the presence of genistein. We have also seen that phosphorylation of LHC II on both tyrosine and threonine residues is decreased by incubation of thylakoids with genistein before phosphorylation.

At low concentrations, genistein is a highly specific tyrosine kinase inhibitor. The concentration of genistein giving half maximal inhibitory effect (IC_{50}) on tyrosine kinase activity is reported to be between 3 μm and 30 μm (Akiyama et al. 1987; Akiyama and Ogawara 1991). At higher concentrations, genistein also inhibits protein histidine kinases with an IC_{50} of 110 μm (Huang et al. 1992) and serine/threonine protein kinases with an IC_{50} of more than 370 μm (Akiyama et al. 1987; Akiyama and Ogawara 1991). In our study, the observed IC_{50} for genistein inhibition of both threonine and tyrosine phosphorylation of LHC II phosphorylation and the light state 2 transitions was found to be approximately 15 μm. The observed IC_{50} was, therefore, at a concentration of genistein substantially lower than those concentrations expected to have any direct effects on protein serine/threonine kinases or protein histidine kinases. Based on the observed inhibitory effects of genistein on the light state 2 transitions (Figures 2 and 3) and the LHC II phosphorylation reactions (Figure 1) we suggest that tyrosine kinase activity is necessary for

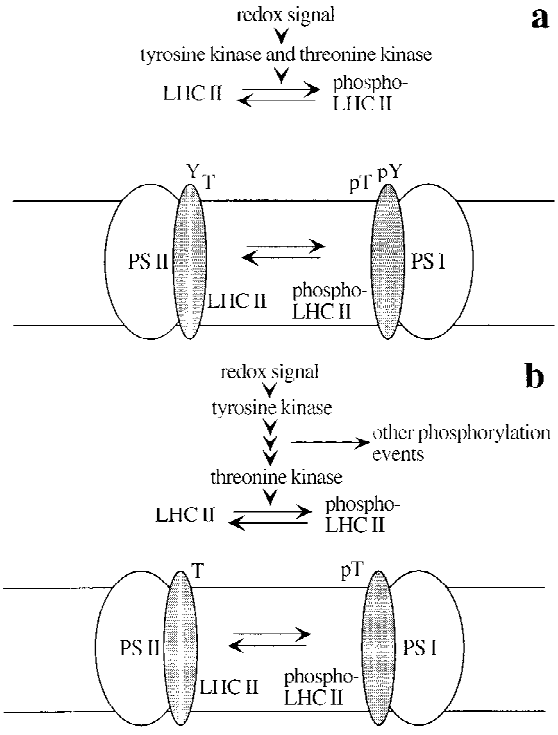

Figure 4. Possible explanations of the requirement for tyrosine kinase activity in the light state 2 transition. The tyrosine kinase activity required for the light state 2 transition to occur (a) a direct phosphorylation on LHC II tyrosine residues (b) in a signalling cascade starting with redox activation of a tyrosine kinase which subsequently activates the kinase phosphorylating LHC II.

plants to adapt to changing light environments by the process of light state 2 transitions. In Figure 4, we outline two models for how tyrosine kinase activity might be required for light state 2 transitions.

In the first model (Figure 4a) the assumption is that the tyrosine phosphorylation required for state 2 transitions to occur takes place on one or more LHC II-tyrosine residues. It is plausible that phosphorylation on Thr-6 is not enough to break up the LHC II trimers and to facilitate dissociation of phospho-LHC II from PS II. There is evidence for the presence of additional phosphorylation sites on LHC II (Dilly-Hartwig et al. 1998) A series of truncated versions of LHC II (Lhcb1) lacking the phosphorylation site Thr-6, was still phosphorylated by a PS II core complex preparation. Not until deletion of the first 58 amino acids, the complete amino terminal loop, did the protein fail to become phosphorylated. There are several tyrosine residues on LHC II exposed on both the stromal and the luminal side of the thylakoid membrane. In the carboxyl and amino terminal loops, both reported to be involved in interactions between LHC II monomers (Kühlbrandt 1994), there are tyrosine residues which can be considered as potential phosphorylation sites. A modification by phosphorylation of one or more such tyrosine residues in a region involved in stabilising the LHC II trimers, could in addition to the modification of Thr-6, be required for the necessary structural changes responsible for the division of the trimers to occur. The trimer break-up in turn facilitates the migration of phospho-LHC II towards PS I, the state 2 transition. It is difficult to identify which residue or residues might be targets for a tyrosine kinase on sequence information alone, since no clearly-defined consensus recognition sequence is known for tyrosine kinases (Kemp and Pearson 1990; Pearson and Kemp 1991).

If the model in Figure 4a is correct, it is also possible that tyrosine phosphorylation precedes the phosphorylation on Thr-6, since tyrosine phosphorylation could be required for exposing Thr-6 or making it more accessible to the kinase responsible for its phosphorylation. The threonine kinase might be unable to recognise LHC II as a substrate without LHC II having a specific tyrosine residue modified. Incubation with genistein would then not only inhibit the tyrosine kinase but also prevent the following threonine phosphorylation and the light state 2 transition, as observed in this study.

In the alternative model (Figure 4b), tyrosine phosphorylation of a protein other than LHC II is required at the beginning of a signalling cascade which leads to the activation of downstream kinases such as the kinase responsible for phosphorylation of LHC II. It has previously been proposed that redox-dependent thylakoid protein phosphorylation in thylakoids is initiated by the activation of a tyrosine kinase which subsequently activates serine/threonine kinases through phosphorylation (Tullberg et al. 1998). Several examples of tyrosine kinases that activate cascades of serine/threonine phosphorylation are known (Johnson and Vaillancourt 1994). Inhibition of tyrosine kinase activity in such a signalling cascade would inhibit activation of the downstream LHC II phosphorylation and, therefore, the transition to light state 2. The phosphorylation of other thylakoid proteins such as the D1 and D2 proteins of the PS II reaction center was in this study also found to be inhibited by genistein (results not shown). The serine/threonine kinase responsible for D1 and D2 phosphorylation might be under the control of the same tyrosine kinase as LHC II.

The tyrosine kinase activity that is implicated in the state 2 transition may be a property of a specific protein tyrosine kinase, or of a dual-specificity kinase, able to catalyse phosphorylation both on tyrosine residues and on serine or threonine residues. Furthermore, tyrosine phosphorylation of LHC II (Figure 4a) and tyrosine phosphorylation of an upstream regulatory component, separate from LHC II (Figure 4b), are not necessarily mutually exclusive. Further experiments are required to distinguish between these possibilities.

We suggest that tyrosine phosphorylation in the transition to light state 2 may be an important, additional factor in regulation of photosynthesis. The mechanism, function, and structural effect of this tyrosine phosphorylation require further investigation. It will also be useful to explore more fully the relationship between tyrosine phosphorylation and threonine phosphorylation of LHC II and other chloroplast proteins.

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