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Selective inhibition of the spinach thylakoid LHC II protein kinase

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Treatment of spinach thylakoids with the adenosine affinity inhibitor 5′-*p*-fluorosulfonylbenzoyl adenosine (FSBA) resulted in at least 95% inhibition of phosphorylation of the light-harvesting protein complex of Photosystem II (LHC II), while the M_r 10000 polypeptide showed a 35% decrease in phosphorylation. This residual kinase activity after FSBA treatment appears to have the same properties as the control, since phosphorylation of the M_r 10000 polypeptide subsequent to FSBA treatment could be achieved with either light or reducing conditions in the dark. [^{14}C]FSBA labelled several polypeptides, but only the M_r 50000 band was protected against the label by prior addition of ADP or adenosine, making it a possible candidate for the LHC II kinase. FSBA had no effect on electron transport, and [^{14}C]FSBA did not label LHC II or the M_r 10000 polypeptide, indicating that the FSBA was not interfering with activation of the kinase or modifying the substrates, but rather acting at the level of the LHC II protein kinase. Inhibition of LHC II phosphorylation by FSBA resulted in the elimination of the slow ATP-induced decrease in variable fluorescence, a parameter believed to be associated with phosphorylation of the LHC II. The half-times and time-course for inhibition of LHC II phosphorylation and inhibition of the ATP-induced decrease of fluorescence yield were identical, consistent with the concept that LHC II phosphorylation plays a major role in this fluorescence change.

Introduction

Spinach chloroplast thylakoids contain membrane-bound kinase activity that is activated by light or reducing conditions in the dark [1–3]. Based on the inhibitory effect of DCMU [1,4], lack of inhibition by the plastoquinone analogue DBMIB [3,5], and the midpoint potential of kinase activation under defined redox conditions [6,7], it is thought that activation results from the reduction of the plastoquinone pool.

The most prominently phosphorylated membrane polypeptides are the light-harvesting chlorophyll pigment protein complex (LHC II) and a polypeptide whose mobility on SDS-polyacrylamide gels corresponds to an M_r electrophoresis 10000 [1,2,8]. The function and identity of the M_r 10000 polypeptide remains to be established, but recent work provides evidence that it is neither the 8 kDa DCCD-reactive subunit of the energy-coupling complex [9] nor cytochrome *b*-559 [10]. It has been suggested that the phosphorylation of

Abbreviations: PS I, Photosystem I; PS II, Photosystem II; LHC II, light-harvesting pigment-protein complex associated with PS II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ado, adenosine; SDS, sodium dodecyl sulfate; FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; FSB, *p*-fluorosulfonylbenzoic acid; DQH_2, durohydroquinone; Me_2SO, dimethylsulfoxide; PPO, 2,5-diphenyloxazole; PMSF, phenylmethylsulfonyl fluoride; Chl, chlorophyll; P_i, inorganic phosphate.

LHC II offers a regulatory mechanism for the State I–State II transition, whereby the distribution of light energy between PS II and PS I is changed to maximize the rate of non-cyclic electron transport [6,11]. In agreement with this hypothesis, it has been shown that protein phoshorylation leads to a decreased rate of PS II electron transport accompanied by increased PS I activity [12], decreased PS II room-temperature variable fluorescence, and increased low-temperature PS I fluorescence (Refs. 6, 11, 13; for a different viewpoint, see Ref. 14).

An unresolved question concerns the number of kinases in the thylakoid membrane and their substrate specificities. Lin et al. [15] have purified two distinct kinases from thylakoids, but neither showed a redox requirement or was able to phosphorylate purified LHC II. In the same work, it was further demonstrated that a crude fraction could be prepared that was capable of phosphorylating purified LHC II [16]. This makes it clear that there is not one single kinase, but at least three. Work by Millner et al. [17] demonstrated that the light-activated kinase activity was severely inhibited by sulfhydryl-directed reagents such as *N*-ethylmalemide. These authors also suggested that there may be two different light-activated kinases responsible for the phopshorylation of LHC II and the M_r 10000 polypeptide, based on the fact that ATP added prior to *N*-ethylmaleimide was able to protect the phosphorylation of LHC II, but not of the M_r 10000 polypeptide [17]. Markwell [18,19] reached a similar conclusion based on the ability of zinc ions to stimulate the phosphorylation of LHC II at low ATP concentrations, while having less effect on the phosphorylation of the M_r 10000 polypeptide.

Here we will attempt to distinguish two kinase activities using the nucleotide affinity inhibitor 5′-*p*-fluorosulfonylbenzoyladenosine. Since its introduction by Wyatt and Colman [20], FSBA has been used successfully in several systems as an affinity labelling reagent for nucleotide binding sites. The covalent interaction of FSBA with nucleotide binding sites has allowed determination of the amino-acid sequence of the allosteric site in sheep heart phosphofructokinase [21], of the nucleotide binding site of the catalytic subunit of the cAMP-dependent protein kinase [22], identification of the catalytic subunit of casein kinase II [23], and association of the kinase activity of the epidermal growth factor receptor with the receptor itself [24]. FSBA has been shown to bind competitively with adenine nucleotides at their binding sites [25] and to modify covalently primarily lysines, although the sulfonyl-fluoride group can also derivatize tyrosine, histidine or cysteine [20].

This work demonstrates that FSBA treatment selectively inhibits the phosphorylation of LHC II, resulting in the elimination of the slow ATP-induced decrease in variable fluorescence. Using [^{14}C]FSBA, we have identified a nucleotide binding polypeptide that is a possible candidate for the LHC II kinase.

Experimental procedures

Chloroplast preparation. Spinach (*Spinacea oleracea* L. cv, Longstanding Bloomsdale) was grown with a 14 h light cycle in a controlled environment chamber at light and dark temperatures of 20°C and 18°C, respectively. Chloroplasts were prepared by the method of Ort and Izawa [26] and suspended in 0.2 M sucrose/5 mM Hepes-NaOH (pH 7.5)/2 mM $MgCl_2$/0.05% (w/v) defatted bovine serum albumin or reaction medium/0.1 M sucrose/50 mM Hepes-NaOH (pH 7.5) 10 mM KCl/5 mM $MgCl_2$/5 mM NaF.

Chloroplast fluorescence and fluorescence-yield changes. Fluorescence was measured as described [27], except that the low-intensity-modulated 645 nm exciting light was passed through a Balzers B-40 (transmittance maximum, 650 nm) interference filter and a 20% transmittance neutral-density filter. The fluorescence was defined by Corning 7-54 and 2-64 blocking filters, and the DC actinic light (14 $J \cdot m^{-2} \cdot s^{-1}$) by a Balzers B-40 (650 nm transmittance maximum) interference filter. Fluorescence yield changes caused by addition of ATP were measured at 20°C in a stirred, water-jacketed, 1 cm square quartz cuvette using chloroplasts (10 μg Chl/ml) in 2.5 ml of reaction medium. The chlorophyll concentration of each sample was determined before and after pretreatment with FSBA prior to fluorescence measurements. Prior to measurement of F_0 fluorescences, nigericin was added to a final concentration of 1 μM to eliminate fast ATP-induced changes in

fluorescence yield. The actinic light was activated, and, after F_{max} was achieved, ATP was added to a final concentration of 1 mM.

Electron-transport measurements. Electron-transport rates were measured with a Clark-type oxygen electrode illuminated by saturating white light passed through a $CuSO_4$ heat filter using chloroplasts (30 μg Chl/ml) suspended in reaction medium with 0.5 mM methyl viologen as the electron acceptor. Where indicated, gramicidin was added to the O_2 electrode chamber to a final concentration of 2 μM. The chlorophyll concentration of each sample was determined before and after pretreatment.

Pretreatment of chloroplasts with 5'-p-fluorosulfonylbenzoyladenosine. FSBA, or an equivalent volume of methanol as the control, was added to the stirred chloroplast suspension (400 μg Chl/ml in reaction medium) in the dark, and allowed to react at 25°C for 30 min. The final FSBA concentration was 1 mM. After this pretreatment, 200 μg aliquots of chlorophyll were transferred to ice-cold 1.5 ml Eppendorf tubes and sedimented by centrifugation for 2 min in a Fischer micro-centrifuge, effectively removing unreacted FSBA and other added nucleotides. Any nucleotide left in the pellet after centrifugation was then further diluted when the samples were suspended in 1.0 ml reaction medium for protein phosphorylation or other measurements.

^{32}P incorporation into thylakoid polypeptides. Chloroplasts equivalent to 200 μg chlorophyll were suspended in 1 ml of reaction medium in a thermostated cell at 19°C. [γ-^{32}P]ATP was added to a specific activity of 30 μCi/μmol with a final ATP concentration of 1 mM. The suspension was illuminated with white light ($2.5 \cdot 10^3$ J · m^{-2} · s^{-1}) for 10 min through a $CuSO_4$ heat filter solution. Following illumination, the chloroplasts were added to 0.1 ml of 35% (v/v) ice-cold perchloric acid and sedimented in the micro-centrifuge for 1 min. The membrane pellet was then washed three times by adding 1 ml 2% (v/v) perchloric acid, sonicated in a Bransonic 12 bath sonicator, and again sedimented. The membranes were then washed twice in 100% ice-cold acetone prior to solubilization in 100 μl of 10% SDS (w/v), neutralized with NaOH, and sonicated to facilitate solubilization. Aliquots of the solubilized chloroplast proteins were taken for determination of protein concentration and ^{32}P incorporation. Incorporation was measured in a Searle-Autocap Scintillation counter using Tritosol [28] scintillation cocktail.

Labelling of chloroplast polypeptides for electrophoresis. Chloroplast proteins were phosphorylated using [γ-^{32}P]ATP. Membranes were washed and solubilized as described above. [^{14}C]FSBA-labelled chloroplasts were prepared as described above, except they were suspended directly into reaction medium. Aliquots (45 μl) of [^{14}C]FSBA in ethanol were added to Pierce 1 ml Reacti-Vials and evaporated to dryness under N_2. The [^{14}C]FSBA (45 mCi/mmol, final concentration 40 μM) was resuspended in 10 μl of methanol, and chloroplasts equivalent to 200 μg chlorophyll and reaction buffer were added to a final volume of 0.5 ml. 40 μM FSBA inhibited LHC phosphorylation by 40%. To test for protection against FSBA, samples were incubated in separate tubes in the presence of ADP or adenosine prior to transfer to Reacti-Vials containing [^{14}C]FSBA. Chloroplasts were then stirred at 25°C for 60 min in the presence of [^{14}C]FSBA. The samples were then transferred to ice-cold 1.5 ml Eppendorf tubes, centrifuged for 2 min in the micro-centrifuge, washed twice with reaction buffer, once with distilled H_2O, twice with ice-cold 100% acetone, then solubilized in 75 μl of 10% SDS (w/v), sonicated, and heated to 50°C for 5 min to insure complete solubilization.

SDS-polyacrylamide gel electrophoresis. Electrophoresis of solubilized membrane proteins was carried out on 15% polyacrylamide slab gels in the presence of 6 M urea according to a modified method of Laemmli [29]. Prior to electrophoresis, an equal volume of a solution containing 4% SDS (w/v)/2% β-mercaptoethanol (v/v)/10% glycerol (v/v)/6 M urea/50 mM Tris (pH 8.9) was added to samples already dissolved in 10% SDS. The proteins were electrophoresed for 18 h at a current of 10 mA.

Treatment of gels following electrophoresis. Following electrophoresis, gels were stained with Coomassie brilliant blue and destained. The gels containing ^{32}P-labelled proteins were divided into lanes and sliced into 3-mm segments, which were then digested in 1 ml 30% H_2O_2 at 85°C for 6 h. After cooling, samples were counted in 10 ml of

Tritosol [28] Scintillation Cocktail.

Fluorograms. Gels containing [^{14}C]FSBA labelled proteins were dehydrated by soaking in Me_2SO, and then in an Me_2SO/PPO mixture [30] prior to drying. After drying, the gels were overlaid with Kodak XAR-5 X-ray film and placed in a Kodak X-omatic cassette with regular intensifying screens. The film was exposed for 3 weeks at −77°C, and developed using a Kodak X-omatic M20 processor.

Autoradiography. Gels containing ^{32}P-phosphorylated proteins were placed in a sealed plastic bag inside a Kodak X-omatic cassette with regular intensifying screens, overlaid with Kodak XAR-5 X-ray film, and exposed at 4°C.

Protein and chlorophyll determination. Protein and chlorophyll concentrations were estimated, respectively, by the methods of Lowry et al. [31] and Arnon [32].

Unlabelled and labelled reagents. [^{14}C]FSBA and ^{32}P were purchased from New England Nuclear, and FSB from Aldrich. FSBA was synthesized as described by Wyatt and Coleman [20], or purchased from Sigma, and [γ-^{32}P]ATP was synthesized as described previously [17].

Results

Inhibition of LHC phosphorylation

The inhibition of protein phosphorylation as a function of incubation time in FSBA shows that a maximum of about 80% inhibition was attained in approx. 30 min, while the control samples show only a small (approx. 9%) decrease in activity (Fig. 1). Analysis of the effect of treatment with FSBA on the phosphorylation of individual proteins revealed that phosphorylation of LHC II was inhibited by at least 95%, while the M_r 10000 polypeptide, which is usually the second-most densely-labelled protein, showed a 35% decrease in incorporation (Fig. 2A and B). The addition of 3 mM ADP or AT prior to FSBA treatment conferred protection against the inhibition of protein phosphorylation by FSBA, with ADP being slightly more effective than ATP (Fig. 2C and D). ADP or ATP added to protect against FSBA inhibition were removed by centrifugation and dilution prior to the phosphorylation to minimize their contribution to overall inhibition of protein phosphorylation.

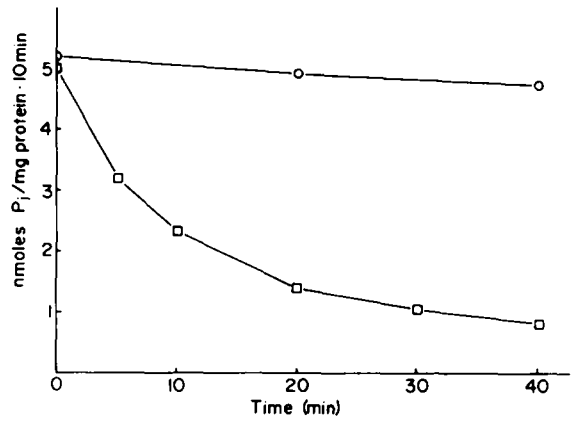

Fig. 1. Time-course of FSBA inhibition of protein phosphorylation. Chloroplasts were diluted into reaction mix to a final concentration of 400 μg Chl/ml. FSBA (□), or an equivalent volume of methanol as a control (○), was added to the stirred chloroplast suspension in the dark and allowed to react at 25°C for the time indicated. At the indicated times an aliquot of chloroplasts (200 μg Chl) was transferred to an ice-cold Eppendorf tube, sedimented, and samples then suspended in 1.0 ml of reaction medium for assay of light-activated protein phosphorylation. Conditions for protein phosphorylation are described in the Section Experimental procedures.

Phosphorylation of the M_r 10000 polypeptide in FSBA-treated chloroplasts was examined. The conditions leading to phosphorylation of the M_r 10000 polypeptide were unchanged by FSBA treatment (Fig. 3). Phosphorylation required light (Fig. 3, compare lanes A–C), or reducing conditions in the dark (Fig. 3, compare lanes C, F and G). The kinase activation by light was sensitive to DCMU (Fig. 3, lane D), and insensitive to DBMIB (Fig. 3, lane E).

The lack of an inhibitory effect of FSBA on electron transport indicates that FSBA did not act to block kinase activation through an effect on electron transport (Table I). FSBA did uncouple electron transport to a small extent, although this uncoupling could be shown not to inhibit kinase activity, since adding the uncouplers nigericin or gramicidin induced a small (20–30%) stimulation of kinase activity rather than an inhibitory effect (Table II). Although others have reported that uncoupler has no effect on protein kinase activity [2,11], the small stimulatory effect was routinely observed in the present studies with either nigericin or gramicidin using freshly prepared, tightly coupled chloroplasts.

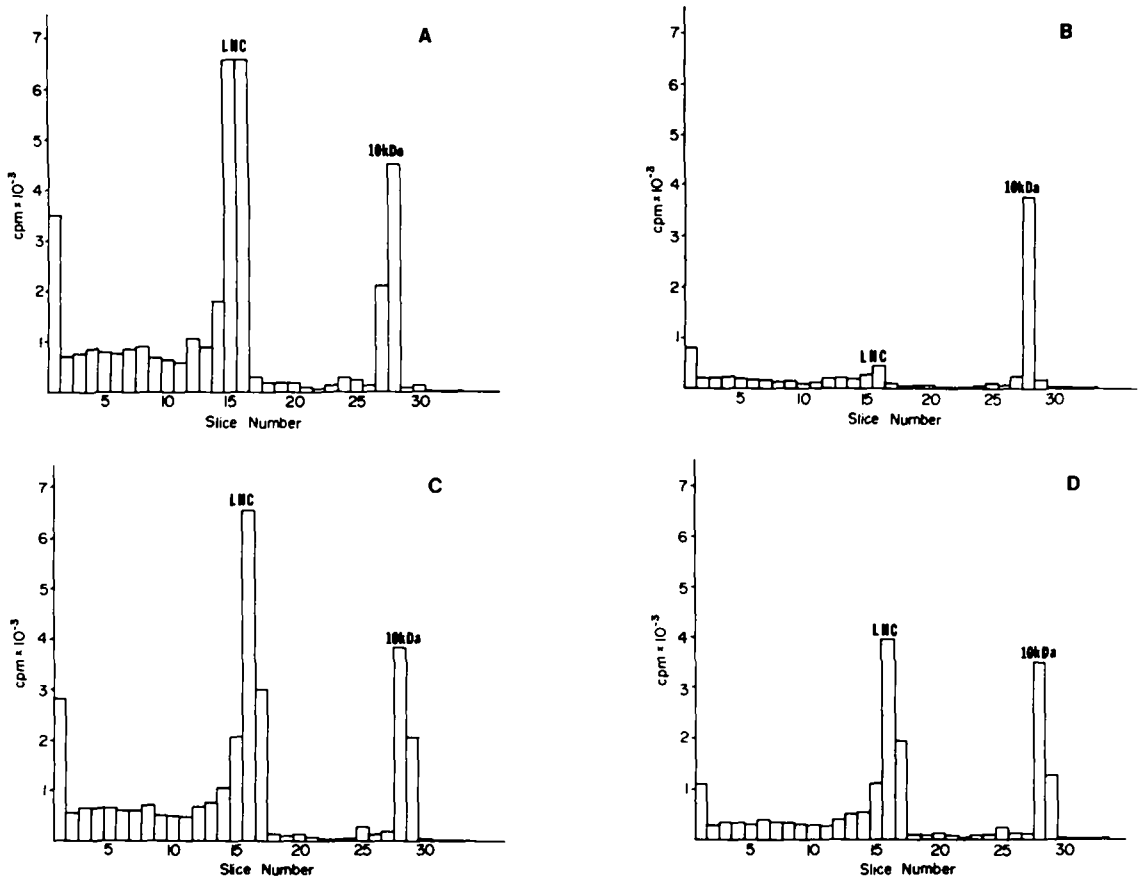

Fig. 2. SDS gel profile of phosphorylated thylakoid proteins after treatment with FSBA in the presence and absence of adenine nucleotides. FSBA treatment conditions were as described under Experimental procedures. (A) Control chloroplasts; (B) chloroplasts treated with FSBA; (C) chloroplasts incubated with 3 mM ADP for 10 min prior to addition of FSBA; (D) chloroplasts incubated with 3 mM ATP for 10 min prior to addition of FSBA. Total protein loaded for each sample was 150 μg.

Chloroplasts incubated for an identical time with fluorosulfonyl-benzoic acid (FSB), a compound that has the same reactive moiety, but is missing the adenosine group, showed no inhibition of LHC II kinase activity (Table III), but a slight stimulation was observed. Addition of 3 mM ADP prior to FSB did not block this slight stimulation, suggesting that the FSB was acting either at some site on the kinase away from the nucleotide binding site or at some site other than the kinase itself.

TABLE I

EFFECT OF FSBA ON ELECTRON TRANSPORT

Conditions of treatment with FSBA were as described in the Section Experimental procedures. Following FSBA treatment, samples were resuspended in 0.1 M sucrose/50 mM Hepes (pH 7.5)/10 mM KCl/5 mM $MgCl_2$/and 5 mM NaF, prior to measuring electron-transfer rates.

Treatment	Rate (μequiv. e^- per mg Chl per h)
Control	140 ± 1
FSBA	165 ± 1
Control (+ gramicidin)	1445 ± 50
FSBA (+ gramicidin)	1405 ± 25)

FSBA inhibition resulting from treatment in the dark was compared to that obtained in the light to determine whether the activated form of the enzyme might be more or less susceptible to the inhibitor, and to determine whether the endogenous substrates could protect against FSBA under activating conditions (Table IV). The results show no significant difference between the amount of inhibition obtained in light or samples treated in the dark.

Two possible explanations of the FSBA effect

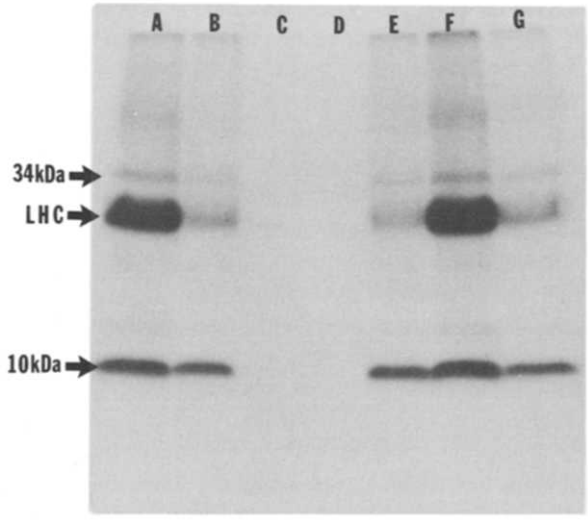

Fig. 3. Effect of FSBA treatment on kinase activation leading to phosphorylation of the M_r 10000 phosphoprotein. Conditions of FSBA treatment were as described in the Section Experimental procedures. Dark redox activation (10 min) was achieved using 0.5 mM DQH_2 as the reductant. DQH_2 was prepared immediately prior to use [38]. (A) Control, light-activated; (B) FSBA-treated, light-activated; (C) FSBA-treated, incubated in the dark in the presence of [^{32}P]ATP; (D) FSBA-treated, light-activated with 5 μM DCMU added prior to phosphorylation; (E) FSBA-treated, light-activated with 3 μM DBMIB added prior to phosphorylation; (F) Control, redox-activated in the dark in the presence of 5 μM DCMU; (G). FSBA-treated, redox-activated in the dark in the presence of 5 μM DCMU. The autoradiogram was exposed for 12 h. Total protein loaded for each sample was 150 μg.

are that the FSBA was acting at the level of the LHC II kinase itself, or by modifying the LHC II, so that it was no longer able to bind to the active site of the kinase. Covalent modification of the LHC II by FSBA was ruled out using the ^{14}C-labelled compound. [^{14}]FSBA was added to normal and NaBr-washed [33] chloroplasts and incubated for 60 min. Fig. 4 compares the Coomassie staining of the protein bands (Fig. 4A) to the autoradiogram of the [^{14}C]FSBA (Fig. 4B). The [^{14}C]FSBA labelling pattern shown in Fig. 4B, lanes 1–3 or NaBr-washed chloroplasts (Fig. 4B, lanes 4–6).

TABLE II

EFFECT OF UNCOUPLER AND FSBA ON PROTEIN PHOSPHORYLATION

Conditions of treatment with FSBA were as described in the Section Experimental procedures. Where indicated, 2 μM nigericin was added to the sample immediately prior to light-activated protein phosphorylation.

Treatment	Rate (nmol P_i per mg protein per 10 min)	Change (%)
Control	5.3 ± 0.3	–
Control (+ nigericin)	6.6 ± 0.2	+26
FSBA	1.4 ± 0.2	−73

In addition to ruling out chemical modification of LHC II by FSBA, it was of interest to see if FSBA labelling would identify possible kinase polypeptide(s). The 60 min treatment labelled several polypeptides, but only the M_r 50000 band was significantly protected against labelling by prior addition of ADP or adenosine (Fig. 4B, lanes 2 and 3, and 5 and 6). It is unlikely that the M_r 50000 band is the β subunit of CF_1, since NaBr washing did not decrease the intensity of the FSBA label in this region (compare Fig. 4B, lanes 1 and 4). The effectiveness of the removal of the β subunit by NaBr can be seen in Fig. 4A, but was further tested on two separate, identically treated samples using the Western blot technique [34] and antibody against spinach CF_1. NaBr washing removed more than 95% of the β subunit (data not shown). In addition to CF_1, NaBr removed 75% of the adenylate kinase activity associated with isolated thylakoids (data not shown) allowing us to rule out [^{14}C]FSBA labelling of that protein as well. Based on these facts and the lower M_r values (22000 and 38000) of the previously purified kinases, shown to not act as kinases with LHC II [14], the M_r 50000 band is a possible candidate for the LHC II kinase.

Fluorescence changes

It was recently reported that Zn^{2+} addition to thylakoids at low ATP concentrations stimulated LHC II phosphorylation, but inhibited the slow ATP-induced decrease in variable fluorescence and the increase in low-temperature PS I fluorescence [18], leading to the conclusion that the fluorescence decrease is not the result of LHC II phosphorylation, contrary to the view of several other laboratories [7,35–37]. This was examined more closely in the present work, using FSBA-treated spinach chloroplasts where primarily two poly-

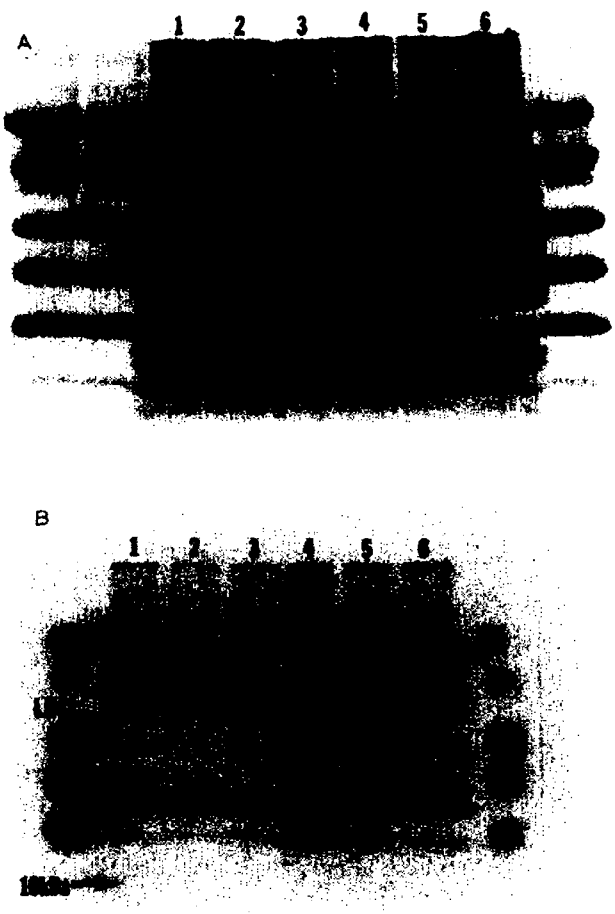

Fig. 4. [^{14}C]FSBA labelling pattern in normal and NaBr-washed chloroplasts. Chloroplasts were prepared as described in Experimental procedures, suspended in reaction medium, and split into two equal aliquots. NaBr was added to one aliquot to a final concentration of 2 M and an equal volume of reaction medium was added to the other. Both samples were stirred on ice for 10 min, diluted 1:1 with ice-cold distilled H_2O, sedimented by centrifugation at $12000 \times g$ for 10 min, resuspended, washed a second time in the same manner, and then resuspended in reaction medium. Aliquots equivalent to 300 μg chlorophyll of normal and NaBr-washed chloroplasts were then pretreated with [^{14}C]FSBA for 60 min at 25°C. The SDS gel profile is shown in (A), and the fluorogram in (B). For the indicated samples, incubation in the presence of 2 mM ADP or Ado was for 5 min in a separate tube before transfer to a vial containing [^{14}C]FSBA. Chloroplasts (1); chloroplasts incubated in 2 mM ADP prior to [^{14}C]FSBA labelling (2); chloroplasts incubated in 2 mM Ado prior to [^{24}C]FSBA labelling (3) NaBr-washed chloroplasts (4); NaBr-washed chloroplasts incubated in 2 mM ADP prior to [^{14}C]FSBA labelling (5); NaBr-washed chloroplasts incubated in 2 mM Ado prior to [^{14}C]FSBA labelling (6). The protein standards bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), β-lactoglobulin (18 kDa), and lysozyme (14 kDa) were marked with radioactive ink after the gel was dried. Ovalbumin was found to run anomalously high (47 kDa) on our gel system and was not included in calculating a standard curve. Other conditions were as described in the Section Experimental procedures. The arrow on the right margin indicates the band corresponding to M_r 50000.

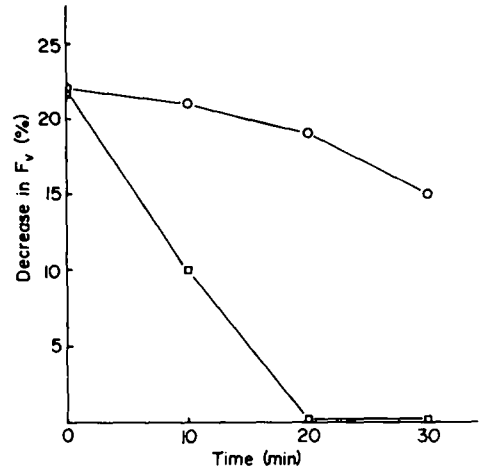

Fig. 5. Effect of FSBA pretreatment on the slow ATP-induced decrease of fluorescence yield. Chloroplasts were incubated with 1 M FSBA (□) or an equivalent volume of methanol (○) at room temperature. Aliquots of chloroplasts (200 μg chlorophyll) were removed at the indicated time, added to an ice-cold 1.5 ml Eppendorf tube and pelleted in a microfuge. Samples were then resuspended in reaction medium and used for fluorescence measurements at a final concentration of 10 μg Chl/ml. Chlorophyll concentration was determined for each sample after pretreatment. 1 μM nigericin was added prior to activating the actinic light and 1 mM ATP was added when F_{max} was achieved. A typical value for F_v/F_0 was 3.0. Other conditions were as described in the Section Experimental procedures.

TABLE III

EFFECT OF FSB TREATMENT ON PROTEIN PHOSHORYLATION

Chloroplasts were diluted into reaction medium to a concentration of 400 μg Chl/ml and treated with 1 mM FSB for 30 min at 25°C. The FSB stock solution in methanol was prepared immediately prior to use. Control chloroplasts were treated identically, but an equal volume of methanol was added rather than FSB. Where indicated, 3 mm ADP was added for 10 min prior to FSB treatment.

Treatment	Rate (nmol P_i per mg protein per 10 min)	Change (%)
Control	5.7 ± 0.1	-
FSB	6.5 ± 0.1	+14
FSB + ADP	6.8 ± 0.4	+19

TABLE IV

COMPARISON OF THE INHIBITION OF PROTEIN PHOSPHORYLATION AFTER FSBA TREATMENT IN ILLUMINATED AND NON-ILLUMINATED SAMPLES

Chloroplasts were either maintained in total darkness or illuminated with $CuSO_4$-filtered white light ($2.5 \cdot 10^3$ J·m^{-2}·s^{-1}) during FSBA pretreatment. Other conditions of FSBA treatment and subsequent light-activated protein phosphorylation were as described in the Section Experimental procedures.

Treatment	Rate (nmol P_i per mg protein per 10 min)	Inhibition (%)
Control, illuminated	4.5 ± 0.4	-
FSBA, illuminated	1.8 ± 0.1	−61
Control, non-illuminated	3.8 ± 0.1	-
FSBA, non-illuminated	1.7 ± 0.1	−54

peptides are phosphorylated, LHC II and an M_r 10000 polypeptide. Blocking the phosphorylation of LHC II by at least 95% with FSBA pretreatment (Fig. 2b) prevented any significant decrease in the slow ATP-induced change in variable fluorescence, but had no effect on the initial or maximum fluorescence levels. It is possible that the residual 5% phosphorylation of LHC II caused some decrease in fluorescence, but the change was not resolvable within experimental error. A time-course for the inhibition of the fluorescence decrease by FSBA as a function of time of pretreatment shows a half-time of 10 min (Fig. 5), which correlates very closely with the half-time for inhibition of LHC II phosphorylation (Fig. 1). This provides direct evidence that the phosphorylation of LHC II plays the major role in the slow ATP-induced fluorescence decrease, with phosphorylation of the M_r 10000 polypeptide having no observable effect.

Discussion

The adenosine affinity inhibitor 5′-*p*-fluorosulfonylbenzoyladenosine was used here to study the number of kinases in the spinach thylakoid membrane and the substrate specificities. It was shown that FSBA-treatment leads to an over 95% inhibition of LHC II phosphorylation, but only 35% inhibition of phosphorylation of the M_r 10000 polypeptide (Fig. 2).

Comparison of the effects of FSB (Table III) and FSBA implies that the adenosine moiety of FSBA directs the inhibitor to the nucleotide-binding site of the LHC II kinase, consistent with the ability of ADP and ATP to protect against FSBA inhibition (Fig. 2). It is possible, however, that the adenosine moiety is essential to target correctly the FSBA to a nucleotide-binding site from which it then differentially affects the polypeptide binding site of a single kinase such that the larger polypeptide, LHC II, is selectively excluded. Further experimentation will be required to rule this out completely, but to our knowledge there is no evidence to indicate that phosphorylation determinants vary with the size of the protein. The observation that the amino-acid determinants for polypeptide recognition and phosphorylation are contained within a linear stretch of 6–10 amino acids [39], combined with the finding that denatured polypeptides are equally good substrates for phosphorylation [40], implies that only a short stretch of the primary sequence is necessary to target a polypeptide correctly to a kinase-active site. Furthermore, the phosphorylation of the minor phosphorylated protein at M_r 34000, like the M_r 10000 phosphoprotein, is only partially inhibited by FSBA treatment (Fig. 3), despite its being larger than the LHC II. Based on these facts it is then unlikely that the inhibition of LHC II phosphorylation by FSBA is the result of formation of a modified kinase polypeptide binding site at some distance from the nucleotide-binding site, such that LHC-II would be selectively excluded based on its greater size.

The inability of [^{14}C]FSBA to label LHC II or the M_r 10000 polypeptide (Fig. 4) allows us to rule out substrate modification as the reason for inhibition of LHC II phosphorylation. Based on the differential inhibition by FSBA and the requirement for reducing conditions for phosphorylation of M_r 10000 polypeptide subsequent to FSBA treatment, we concluded that two different protein kinases act on the LHC II and the M_r 10000 proteins.

Contrary to our findings, data have recently been presented that was interpreted in terms of

evidence for a single kinase [41]. The argument is based on the change in $S_{0.5}$ for ATP as a function of the change in the level of LHC II and M_r 10000 phosphoprotein in intermittent-light-grown peas. This argument can be theoretically correct. However, it is based on the assumption that the level of enzyme(s) remains constant during the developmental process. Since the mature spinach thylakoid is known to have at least three different protein kinases [15,16] (and it is not known when these enzymes appear and how the levels change during development), it is equally possible to explain the data presented by those authors based on changes in the total amount and ratio of two or more kinases.

[^{14}C]FSBA was used to identify nucleotide binding proteins, other than CF_1, that could be possible candidates for the LHC II kinase. A labelling period of 60 min resulted in labelling of several polypeptides, but only the M_r 50000 protein showed significant protection by adenine nucleotides (Fig. 2). It is unlikely that the M_r 50000 protein is the β subunit of CF_1, since the labelling intensity in this region was not diminished by treatment with NaBr, which removes more than 95% of the β subunit of CF_1. We were also able to rule out adenylate kinase as a candidate for the M_r 50000 band, since NaBr removes 75% of that activity as well as the previously purified kinases based on the large difference in M_r values.

Phosphorylation of LHC II has been proposed to be an in vivo mechanism which leads to the well-characterized State I–State II transition (reviewed in Ref. 42). It is thought that the phosphorylation of LHC II causes this polypeptide to migrate from the PS-II-enriched appressed regions of the membrane to the PS-I-enriched non-appressed or stromal regions, effectively increasing the cross-section for light absorption by PS I [42,43]. Recently, data have been published that would suggest that LHC II phosphorylation plays no role in this process [18]. These authors describe an increase in the phosphorylation of LHC II in thylakoids upon the addition of zinc ions at low ATP concentration that is not accompanied by a decrease in PS II room-temperature fluorescence or any increase in the low-temperature PS I emission at 470 nm. Dilley and Rothstein [44] have shown that Zn^{2+} ions bind tightly to ionizable groups on thylakoids causing extensive shrinkage, while Mg^{2+} and monovalent cations do not similarly affect the thylakoids. It is possible to explain the lack of an effect of phosphorylation on fluorescence observed in the presence of Zn^{2+} ions as the result of tight binding of the positively charged Zn^{2+} with the phosphate on LHC II. Such charge neutralization via a tightly bound ion could effectively block the phosphorylation-induced charge repulsion thought to cause the lateral migration of LHC II, thus eliminating any changes in excitation energy distribution.

Here we demonstrate that inhibition of LHC II phosphorylation by FSBA, which has a comparatively small effect on the phosphorylation of the M_r 10000 phosphoprotein (Fig. 2), blocks the slow ATP-induced decrease in variable fluorescence (Fig. 5). The time-course for the inhibition of the change in fluorescence corresponds very well with that for inhibition of LHC II phosphorylation, with both having a half-time of 10 min. We conclude that phosphorylation of the LHC II plays the major role in the phosphorylation-induced decrease in PS II room temperature fluorescence, and that the M_r 10000 phosphoprotein plays no detectable role in this phenomenon. Since there are only two major phosphorylated proteins in spinach thylakoids under our experimental conditions, LHC II and an M_r 10000 polypeptide, with a minor band of variable intensity at M_r 34000, the phosphorylation of which is only partially inhibited by FSBA treatment (Fig. 3), it is clear that LHC II phosphorylation is primarily responsible for the decrease in variable fluorescence of PS II.

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