Proc. Natl. Acad. Sci. USA
Vol. 77, No. 9, pp. 5253–5257, September 1980
Botany

Chloroplast phosphoproteins: Regulation of excitation energy transfer by phosphorylation of thylakoid membrane polypeptides

(chlorophyll fluorescence/light-harvesting pigment–protein/photosystems I and II)

JOHN BENNETT*, KATHERINE E. STEINBACK, AND CHARLES J. ARNTZEN

U.S. Department of Agriculture, Science and Education Administration, Department of Botany, University of Illinois, Urbana, Illinois 61801

Communicated by Warren L. Butler, May 27, 1980

ABSTRACT **Incubation of isolated chloroplast thylakoid membranes with [γ-^{32}P]ATP results in phosphorylation of surface-exposed segments of several membrane proteins. The incorporation of ^{32}P is light dependent, is blocked by 3(3,4-dichlorophenyl)-1,1-dimethylurea (diuron, an inhibitor of electron transport), but is insensitive to uncouplers of photophosphorylation. Polypeptides of the light-harvesting chlorophyll *a*/*b*-protein complex are the major phosphorylated membrane proteins. Addition of ATP to isolated chloroplast thylakoid membranes at 20°C results in a time-dependent reduction of chlorophyll fluorescence emission; this is blocked by diuron but not by nigericin. ADP could not substitute for ATP. Chlorophyll fluorescence induction transients showed a decrease in the variable component after incubation of the membranes with ATP. Chlorophyll fluorescence at 77 K of phosphorylated thylakoid membranes showed an increase in long-wavelength emission compared with dephosphorylated controls. We conclude that a membrane-bound protein kinase can phosphorylate surface-exposed segments of the light-harvesting pigment–protein complex, altering the properties of its interaction with the two photosystems such that the distribution of absorbed excitation energy increasingly favors photosystem I.**

The photosynthetic membranes (thylakoid membranes) of plant (1) and green algal (2) chloroplasts contain several polypeptides that are reversibly phosphorylated on surface-exposed threonyl residues (3). The protein kinase that phosphorylates the polypeptides is membrane bound, is dependent on Mg^{2+}, and is activated by light (4). Dephosphorylation is due to a thylakoid-bound phosphatase that is stimulated by Mg^{2+} but is not dependent upon illumination (3). The favored substrates of both enzymes are two polypeptides (24,000 and 26,000 daltons) that are constituents of the light-harvesting chlorophyll–protein complex (LHC) (5, 6). Together these polypeptides account for about 30% of the total intrinsic thylakoid protein. Their function is to organize about half the chlorophyll (Chl) *a* and most of the Chl *b* of the membrane into an antenna complex that absorbs photons and subsequently directs excitation energy to the core complexes of photosystems I and II (PS I and II) (7–9).

Photosynthetic electron transport in chloroplasts is mediated by two light reactions acting in series; maximal efficiency of noncyclic electron transport requires a balanced distribution of absorbed excitation energy to the PS II and PS I reaction centers. It is generally accepted that an *in vivo* regulatory mechanism controls this distribution under varying environmental conditions (10). This regulatory mechanism requires the presence, in chloroplast membranes, of the LHC serving PS II (11). In this paper we present results that show that a physiological role of membrane protein phosphorylation in chloroplasts is to regulate the distribution of absorbed excitation energy between PS I and II.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

METHODS

Plants. Seedlings of dwarf pea (*Pisum sativum* L. var. Progress 9) were grown as described (12). Leaves were harvested from 12- to 20-day-old seedlings.

Materials. High-specific-activity [γ-^{32}P]ATP was obtained from The Radiochemical Centre (Amersham, England). X-ray-sensitive film (Kodirex) was supplied by Kodak Limited (Hemel Hempstead, Herts, England).

Isolation and Phosphorylation of Thylakoids. Leaves (20 g) were homogenized in 100 ml of 0.35 M sucrose/25 mM Hepes·NaOH, pH 7.6/2 mM Na_4EDTA/2 mM D-isoascorbate by a Polytron (Kinematica Gmbh, Lucerne, Switzerland), and the homogenate was filtered twice through eight layers of muslin that had been washed in water and cold homogenizing medium. The filtrate was centrifuged at 2800 × *g* for 1 min, the pellet was resuspended in 20 ml of 10 mM Tricine·NaOH, pH 7.8/10 mM $MgCl_2$/5 mM 2-mercaptoethanol, and the suspension was left on ice for 5 min to permit osmotic rupture of the chloroplasts. The suspension was centrifuged at 2800 × *g* for 5 min and the pellet was washed by resuspension in 10 ml of resuspending medium followed by a second centrifugation. The final pellet of washed thylakoids was suspended in 0.1 M sorbitol/10 mM Tricine·NaOH, pH 7.8/10 mM $MgCl_2$/1 mM KCl/5 mM 2-mercaptoethanol to a final concentration of 200 μg of Chl per ml.

Portions (500 μl) of the thylakoid suspension were equilibrated for 5 min in an illuminated water bath (20°C; photosynthetically active light of 400–700 nm: 500 $\mu E\ m^{-2}\ sec^{-1}$) in the presence of all indicated additions except ATP. Phosphorylation was initiated by addition of [γ-^{32}P]ATP (100 Ci/mol; 1 Ci = 3.7×10^{10} becquerels) to a final concentration of 200 μM, except for the K_m determination, when 10–600 μM ATP was used. After incubation periods specified in Figs. 1 and 2 and Table 1, proteins were extracted from the membrane and precipitated by addition of acetone (2 ml). For incubation with and without trypsin, two identical samples were incubated for 5 min at 20°C in the light in the presence of 200 μM [γ-^{32}P]ATP and then placed on ice for 5 min. Trypsin was added to one sample (final concentration, 50 $\mu g\ ml^{-1}$). After a further 25 min on ice, material was extracted from the two membrane samples with acetone. The proteins that precipitated out of the acetone solution were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, and the gels were stained, dried, and autoradiographed as described (1, 3). Radioactivity incorporated into the LHC protein doublet (24,000- and 26,000-dalton polypeptides) was determined by excising the stained bands from gels and measuring radioactivity by Cerenkov spectrometry (1).

Abbreviations: LHC, light-harvesting chlorophyll *a*/*b*-protein complex; PS I and PS II, photosystems I and II; Chl, chlorophyll; diuron, 3(3,4-dichlorophenyl)-1,1-dimethylurea.

* Permanent address: Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL West Midland, England.

Fluorescence Measurements. For measurements of steady-state chlorophyll fluorescence and fluorescence inductions at room temperature, membrane samples were suspended in 50 mM Tricine·NaOH (pH 7.8) containing 10 mM $MgCl_2$, 100 mM sorbitol, and a final concentration of 5 μg of Chl per ml. Membranes were preincubated in the dark for 10 min. Inductions were recorded as described (12).

Low-temperature fluorescence emission spectra were measured at 77 K by the dual-channel ratiometric acquisition method with a System 4000 scanning polarization spectrofluorimeter (SLM Instruments, Urbana, IL). Samples were rapidly frozen in 0.5-mm (inner diameter) capillary tubes in 50 mM Tricine·NaOH, pH 7.8/10 mM $MgCl_2$/50% (vol/vol) glycerol.

RESULTS

Thylakoid Protein Phosphorylation. In the presence of 200 μM [γ-^{32}P]ATP and 10 mM $MgCl_2$, isolated illuminated pea thylakoids incorporated radioisotope into several membrane polypeptides (Fig. 1). Two polypeptides of the LHC (24,000 and 26,000 daltons) were the most heavily labeled phosphoproteins.

Phosphorylation of the LHC polypeptides did not follow a linear time course (Fig. 2*A*). During the first minute of sample illumination, phosphorylation was rapid, but thereafter the additional amount of ^{32}P incorporation declined; during the 5- to 10-min period, the rate was only 20% of the initial rate.

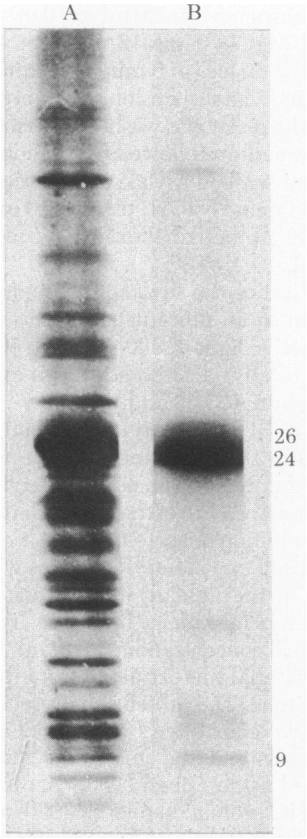

FIG. 1. Phosphorylation of thylakoid polypeptides. Isolated washed pea thylakoids were illuminated in the presence of 200 μM [γ-^{32}P]ATP for 5 min. Thylakoid proteins were precipitated with acetone and fractionated by NaDodSO$_4$/polyacrylamide gel electrophoresis. Track A, stained gel; track B, autoradiogram. The stained bands designated 26 and 24 are the major and minor polypeptide components, respectively, of LHC (26,000 and 24,000 daltons, respectively). They are approximately equally labeled with ^{32}P. The other phosphorylated proteins have not been identified.

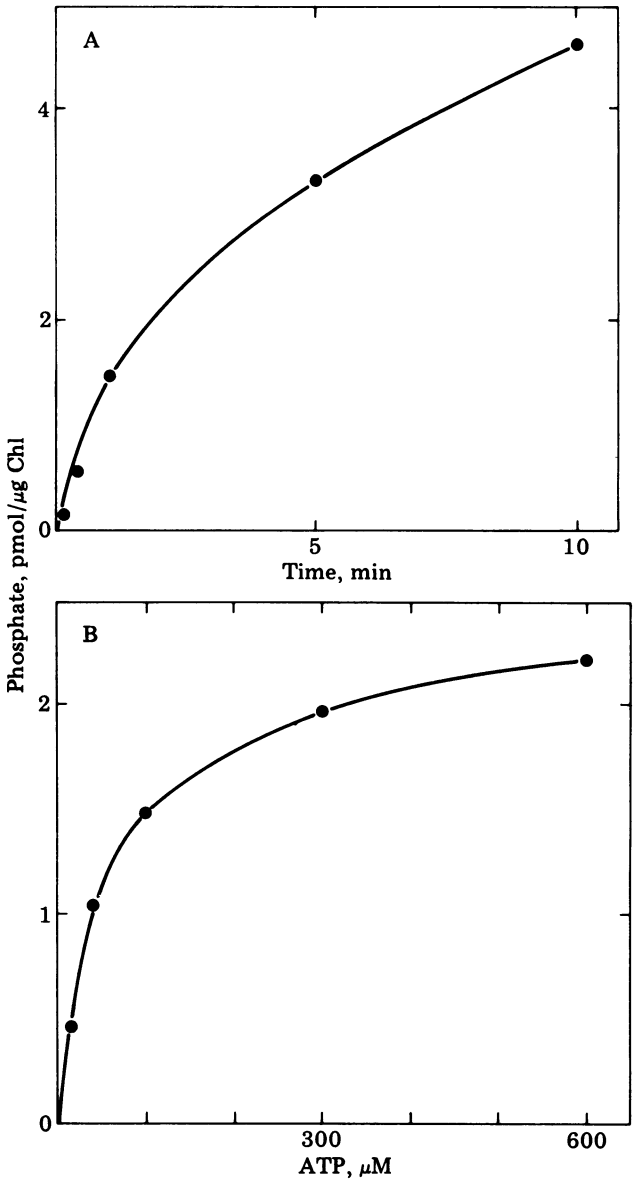

FIG. 2. Phosphorylation of the two LHC polypeptides. (*A*) Time course up to 10 min in the presence of 200 μM ATP. (*B*) ATP dependence (up to 600 μM) in a 2-min assay. Data presented are from scintillation spectrometry of LHC polypeptides excised from polyacrylamide gels.

The decline could have been due to a number of factors, including inactivation of the kinase, reduction in the number of sites remaining to be phosphorylated, and comcomitant dephosphorylation by the phosphoprotein phosphatase.

The rate of phosphorylation was dependent on the ATP concentration. Fig. 2*B* shows the extent of phosphorylation of the LHC polypeptides over a 2-min assay period in the presence of 10–600 μM ATP. A short assay period minimized the effect of the nonlinear time course and ensured that the measured rates were as close as possible to initial velocities. Double-reciprocal plots of data such as that in Fig. 2*B* gave an apparent mean ($\pm$ SD) K_m for ATP of 90 $\pm$ 20 μM.

The effects of 3(3,4-dichlorophenyl)-1,1-dimethylurea (diuron, an inhibitor of photosynthetic electron transport) and of nigericin and gramicidin D (uncouplers of photophosphorylation) on light-dependent LHC phosphorylation are reported in Table 1. In this experiment the assay period was 5 min, and the data reported are specifically for phosphorylation of the

Table 1. Thylakoid protein phosphorylation requires photosynthetic electron transport

Conditions of incubation	Phosphorylation, %
Light (zero time)	6
Dark (5 min)	2
Light (5 min)	100
+ Diuron (10 μM)	11
+ Nigericin (1 μM)	104
+ Nigericin (10 μM)	86
+ Gramicidin D (0.1 μM)	82
+ Gramicidin D (1 μM)	90
Followed by 30 min at 0°C	63
Followed by 30 min at 0°C + trypsin (50 μg/ml)	5

Isolated pea thylakoids were incubated with [γ-^{32}P]ATP in the presence or absence of diuron, nigericin, or gramicidin D. Phosphorylated thylakoids were also incubated at 0°C with and without trypsin. Phosphorylated polypeptides were fractionated by gel electrophoresis and the level of phosphate incorporated into LHC (24,000- and 26,000-dalton polypeptides) was determined. The control incorporation (5-min incubation in the light in the absence of inhibitors) in this experiment was 5.0 pmol of ^{32}P per μg of Chl. The other results are expressed as a percentage of this control. In other experiments, with fully dephosphorylated membranes as starting material, up to 20 pmol of ^{32}P per μg of Chl was incorporated into membranes under optimal conditions.

LHC polypeptides. Phosphorylation was clearly light dependent but did not require photophosphorylation or formation of a pH gradient (which would be blocked by nigericin or gramicidin D) for activation of the latent protein kinase. Diuron inhibited the kinase activation by 90%. Uncouplers did inhibit ^{32}P labeling of thylakoid polypeptides in isolated, illuminated, intact chloroplasts supplied with [^{32}P]orthophosphate (1). In this case, photophosphorylation is required to generate [γ-^{32}P]ATP within the intact organelle from endogenous ADP.

Incubation of phosphorylated membranes with trypsin at 0°C for 30 min led to an almost total loss of radioactivity from the membrane (Table 1). We have previously shown that for the LHC polypeptides, trypsin treatment results in cleavage of 10 amino acids from the surface-exposed segment of each chain (6). These surface-exposed segments carry the phosphorylation sites (3, 6). Incubation in the absence of trypsin led to a slight loss of radioactivity (Table 1). This effect has been reported previously (3) and probably indicates that the phosphatase is active to some extent even at 0°C.

Room-Temperature Fluorescence. Analysis of Chl fluorescence at room temperature allows characterization of the redox state of the primary electron acceptor for PS II. It also provides information relating to the distribution of absorbed excitation energy between PS I and II (8, 13, 14). When isolated thylakoids were continually illuminated at room temperature in the presence of 10 mM $MgCl_2$, the fluorescence yield over several minutes remained relatively constant (Fig. 3*A*). The addition of ATP (200 μM) led to a rapid decline in fluorescence during the first 20 sec and was followed by a slow decrease that persisted for several minutes (Fig. 3*B*). Nigericin reversed the rapid phase of fluorescence decrease but did not affect the slow fluorescence decline.

Addition of nigericin to thylakoids prior to addition of ATP (Fig. 3*C*) prevented the rapid phase of fluorescence quenching induced by ATP (presumably related to membrane energization and associated conformational changes), yet allowed the slow decline to go to completion with a half-time of about 3 min. This time factor is closely comparable with the measured half-time of LHC protein phosphorylation (Fig. 2*A*).

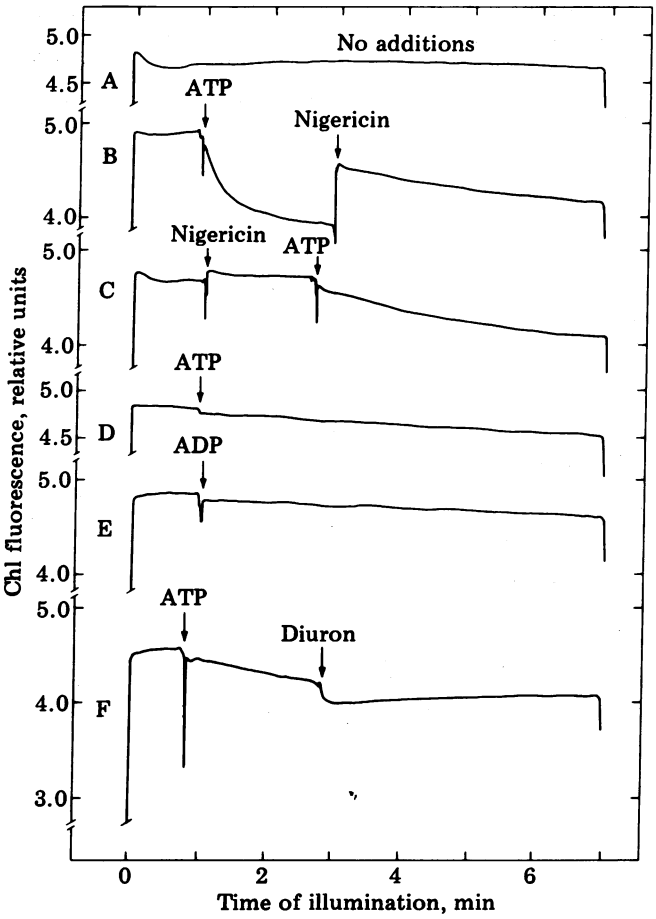

FIG. 3. Room-temperature fluorescence of isolated pea thylakoid membranes suspended in 0.1 M sorbitol/50 mM Tricine·NaOH, pH 7.8/10 mM $MgCl_2$/1 mM KCl. (*A*) No additions; (*B*) 200 μM ATP, then 1 μM nigericin added during illumination; (*C*) 1 μM nigericin added prior to 200 μM ATP addition; (*D*) 1 μM nigericin and 10 μM diuron added prior to illumination, 200 μM ATP added during illumination; (*E*) 1 μM nigericin added prior to illumination, 200 μM ADP added during illumination; (*F*) 1 μM nigericin added prior to illumination, 200 μM ATP added during illumination, followed by addition of 10 μM diuron.

The slow fluorescence decrease induced by ATP resembled protein phosphorylation in its sensitivity to diuron. When ATP was added to thylakoids after addition of nigericin and diuron (Fig. 3*D*), the slow fluorescence decline was no longer observed. When diuron was added after 2 min of incubation in the presence of ATP, a reversal of the slow fluorescence decline was observed (Fig. 3*F*). This reversal is expected because the thylakoid phosphoprotein phosphatase is insensitive to diuron (3). ADP could not replace ATP in causing a fluorescence decrease (Fig. 3*E*). As an unrelated point, diuron decreases the fluoresence yield of the isolated chloroplasts by about 15%; this is consistent with the observations of Vernotte *et al.* (15).

Chl fluorescence induction transients were used to determine if the fluorescence decrease, occurring concomitantly with protein phosphorylation, was a general quenching effect or a dynamic quenching specific for altered functional properties of the light-harvesting pigment bed. For these experiments, chloroplasts were preincubated in reaction solutions to allow protein phosphorylation. At various intervals (see Fig. 4), samples were removed, incubated in the dark (to reoxidize the electron transport chain) for 10 min at 0°C in the presence of 10 mM NaF (to inhibit the endogenous phosphatase), and then analyzed via fast fluorimetry to determine immediate (F_0) and maximal (F_m) fluorescence intensities. In chloroplasts collected

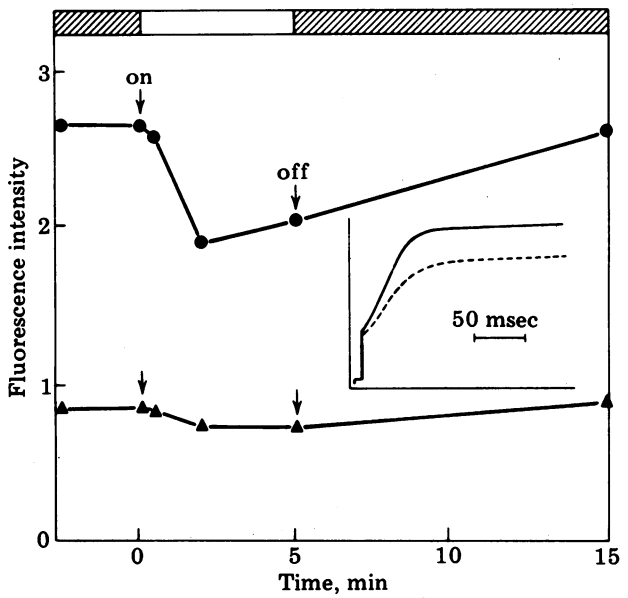

FIG. 4. Analysis of room-temperature Chl fluorescence in chloroplast membrane samples incubated under protein phosphorylating conditions. A 5-ml volume containing chloroplasts (200 μg of Chl per ml) in 50 mM Tricine·NaOH, pH 7.8/100 mM sorbitol/10 mM $MgCl_2$/1 mM KCl/1 μM nigericin was incubated in the dark for 10 min, then illuminated for 5 min (open bar at top of figure) followed by 10 min in the dark. ATP (200 μM) was added to the suspension 2 min prior to illumination. At indicated times, 50-μl chloroplast samples were removed and transferred to ice-cold fluorescence assay medium containing 10 mM NaF. After a 10-min incubation in the dark on ice, diuron was added to each sample to a concentration of 5 μM. Induction transients were acquired with a digital oscilloscope; (*Inset*) tracings of the plotted fast records are shown for dephosphorylated membranes (zero time, ——) and phosphorylated membranes (5 min in the light, - - -). The fluorescence intensity measured within 1 msec of full shutter opening was defined as F_0 (▲); the maximal fluorescence attained after 200 msec of illumination was defined as F_m (●).

during the light treatment, a specific decrease in F_m occurred (Fig. 4); this change was reversed if incubation in the light was followed by a subsequent incubation of the thylakoids in the dark in the absence of NaF, in which the phosphatase is known to be active in dephosphorylating the LHC. In addition, if 20 mM EDTA was added to the chloroplast samples during the dark 2 min prior to analysis, the fluorescence intensity of all samples was reduced to a "low-salt" level (see ref. 16). In these "low-salt" samples, pretreatment conditions allowing protein phosphorylation did not affect F_0 or F_m levels; i.e., protein phosphorylation cannot influence fluorescence under conditions of maximal energy transfer from PS II to I.

At 77 K, the fluorescence emissions of the PS II and PS I pigment beds are clearly distinguished by peak emission near 685–695 and 730–740 nm, respectively (17). Fig. 5 shows 77 K fluorescence emission spectra of thylakoid samples incubated in the presence of ATP in the dark (dephosphorylated) or light (phosphorylated). For these experiments, chloroplast thylakoids were quickly frozen after various pretreatments. After 5 min of incubation in the light, the fluorescence emission arising from PS I (734 nm peak) was enhanced relative to emission from PS II (685 and 695 nm). When the test sample was first illuminated for 5 min and then allowed to incubate in the dark for 10 min (to allow LHC dephosphorylation), the fluorescence emission characteristics reversed to favor emission from PS II. The increased relative fluorescence arising from PS I (734 nm) in the phosphorylated membrane samples indicates an increased energy transfer to the PS I pigment bed at the expense of PS II (17).

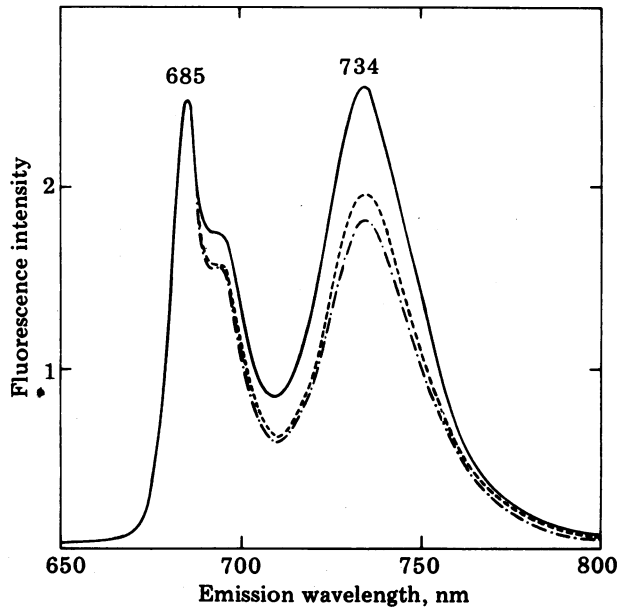

FIG. 5. Low-temperature (77 K) fluorescence emission spectra of chloroplast thylakoid membranes, preincubated (prior to freezing) with 200 μM ATP in the dark or light. - - -, Incubation in the dark for 5 min; ——, incubation in light for 5 min; -·-, incubation in light for 5 min followed by 10 min of incubation in dark. All spectra were acquired in digital form by computer from the spectrofluorometer. The data presented are spectra normalized to display equal fluorescence intensity at 685 nm.

DISCUSSION

In 1969, Bonaventura and Myers (18) demonstrated that the light-harvesting properties of photosynthetic cells adjust to preillumination conditions. The slow (several minutes) state I–state II changes they reported have been interpreted as either a change in the absorption cross section of the antennae serving PS I and II (19) or a change in energy transfer from PS II to I (20). In either case, the physiological advantage to the cells of this regulatory change is the maintenance of balanced input of quanta into the two photosystems acting in series, thus allowing maximal efficiency of photosynthetic electron transport under diverse environmental regimes.

The mechanism by which regulation of photosynthetic light-harvesting properties is achieved has been the object of numerous investigations. It is known that changing cation concentrations in a preparation of isolated thylakoids dramatically alters the energy transfer from PS II to I (13, 14, 17); it has been hypothesized that light-induced cation fluxes across thylakoid membranes may alter local cation concentrations within the chloroplast and thereby influence the light-harvesting pigment bed (for review, see ref. 13). There are several inconsistencies in this model, however (also recognized by previous authors). First, both photosystems can mediate electron transport coupled to cation efflux; it is not clear how the ion fluxes could easily be a selective regulatory process. Second, light-induced cation fluxes observed in isolated membranes are rapid (10–30 sec) whereas the physiological changes in excitation energy distribution appear over a time frame of several minutes (14, 18).

The data presented in this manuscript provide a new hypothesis for the physiological control of photosynthetic light harvesting: phosphorylation of surface-exposed segments of the major thylakoid pigment–protein (LHC) alters its properties such that its interactions with PS II and I are modified. This results in corresponding changes in distribution of absorbed excitation energy.

The data supporting this concept rely on the parallel time course of phosphorylation of the LHC and changes in Chl fluorescence (indicating changing energy distribution). Both phenomena (phosphorylation and fluorescence changes) are dependent upon light and ATP, are insensitive to uncouplers, are blocked by the electron transport inhibitor diuron, and are reversible in the dark when a membrane-bound phosphatase is known to be active (3). The fluorescence quenching occurring at 20°C under conditions of protein phosphorylation is specific for the variable component of fluorescence and is not observed in "low-salt" chloroplasts. These data indicate a dynamic quenching which can be explained by an increased energy transfer from the PS II pigment bed to that of PS I (which has a much lower fluorescence yield at 20°C). Such a change is supported by the increased relative PS I (734 nm) fluorescence at 77 K in phosphorylated samples.

In summary, these data demonstrate that phosphorylation of the surface-exposed segments of LHC polypeptides results in alterations of the chloroplast pigment bed such that there is an increase in excitation energy transfer from PS II to PS I. In contrast, dephosphorylation potentiates cation-dependent inhibition of this energy transfer. The activation of the kinase (which mediates LHC phosphorylation) by photosynthetic electron transport is therefore the central process by which chloroplasts exert physiological control of photosynthetic light-harvesting efficiency.

This research was supported, in part, by National Science Foundation Grant PCM-7718953.

1. Bennett, J. (1977) *Nature (London)* **269,** 344–346.
2. Beliveau, R. & Bellemare, G. (1979) *Biochem. Biophys. Res. Commun.* **88,** 797–803.
3. Bennett, J. (1980) *Eur. J. Biochem.* **104,** 85–89.
4. Bennett, J. (1979) *FEBS Lett.* **103,** 342–344.
5. Bennett, J. (1979) *Eur. J. Biochem.* **99,** 133–137.
6. Mullet, J. E. & Arntzen, C. J. (1979) *J. Cell Biol.* **83,** 1926 (abstr.).
7. Arntzen, C. J. (1978) *Curr. Top. Bioenerg.* **8,** 111–160.
8. Butler, W. L. (1978) *Annu. Rev. Plant Physiol.* **29,** 345–378.
9. Thornber, J. P., Markwell, J. P. & Reinman, S. (1979) *Photochem. Photobiol.* **29,** 1205–1216.
10. Myers, J. (1971) *Annu. Rev. Plant Physiol.* **22,** 289–312.
11. Lieberman, J. R., Bose, S. & Arntzen, C. J. (1978) *Biochim. Biophys. Acta* **502,** 417–419.
12. Burke, J. J., Ditto, C. L. & Arntzen, C. J. (1978) *Arch. Biochem. Biophys.* **187,** 252–263.
13. Barber, J. (1976) in *Topics in Photosynthesis,* ed. Barber, J. (Elsevier/North-Holland, Amsterdam), Vol. 1, pp. 89–124.
14. Williams, W. P. (1977) in *Topics in Photosynthesis,* ed. Barber, J. (Elsevier/North-Holland, Amsterdam), Vol. 2, pp. 99–147.
15. Vernotte, C., Etienne, A. L. & Briantais, J.-M. (1979) *Biochim. Biophys. Acta* **545,** 519–527.
16. Steinback, K., Burke, J. J. & Arntzen, C. J. (1979) *Arch. Biochem. Biophys.* **195,** 546–557.
17. Butler, W. L. (1977) in *Encyclopedia of Plant Physiology,* eds. Trebst, A. & Avron, M. (Springer, New York), Vol. 5, pp. 149–167.
18. Bonaventura, C. & Myers, J. (1969) *Biochim. Biophys. Acta* **189,** 366–383.
19. Wang, R. T. & Myers, J. (1974) *Biochim. Biophys. Acta* **347,** 134–140.
20. Murata, N. (1969) *Biochim. Biophys. Acta* **189,** 171–181.