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GTP-induced chloroplast membrane protein phosphorylation and Photosystem II fluorescence changes: evidence for multiple protein kinase activities

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The ability of GTP to act as a substrate for the endogenous protein kinase(s) present in isolated spinach thylakoids has been investigated. It is shown that GTP acts as a substrate for the protein kinase(s) and the phosphorylated thylakoid membrane proteins have been analysed by SDS-polyacrylamide gel electrophoresis. A comparison of the ATP- and GTP-induced phosphorylation of the polypeptides shows that (a) GTP is a less efficient substrate compared with ATP; (b) all the major phosphoproteins (e.g., LHC-II, 9–10 kDa) labelled in the presence of ATP are also labelled in the presence of GTP; (c) two groups of minor polypeptides are labelled only in the presence of ATP but not in the presence of GTP. These data, in contrast to an earlier study (Millner, P.A., Widger, W.R., Abbott, M.S., Cramer, W.A. and Dilley, R.A. (1982) J. Biol. Chem. 257, 1736–1742) suggest that GTP binds to the active site of the protein kinase(s) which is (are) responsible for the phosphorylation of LHC II and the 9–10 kDa proteins. Furthermore, multiple protein kinases are indicated and at least one of these activities is unable to utilise GTP as a substrate. A comparison of the ATP- and GTP-induced phosphorylation on the fluorescence decline of Photosystem II though shows that the GTP-induced phosphorylation of the LHC II polypeptides fails to elicit a decline as observed with ATP. The results are discussed with respect to different protein kinase activities and correlations between LHC II polypeptide phosphorylation and PS II fluorescence decline.

Introduction

Several chloroplast thylakoid membrane proteins have been shown to be reversibly phosphorylated in vivo and in vitro [1–6] by endogenous protein kinase(s) and phosphatase(s). The activation of the kinase(s) appears to require the reduction of the plastoquinone pool, by light or chemical reduction, while the phosphatases are independent of this regulation. Although at least 20 polypeptides (M_r = 8000–70000) have been reported to be phosphorylated [1] the major phosphoproteins in spinach thylakoids and in other higher plants are the light-harvesting chlorophyll *a/b* binding protein complex (LHC II) polypeptides and the 9–10 kDa polypeptide [3–6]. Furthermore, several protein kinase activities have been identified and three different kinase activities have been purified [7–9].

In view of this complexity it is quite probable that different polypeptides may be phosphorylated by different kinases. Indeed the kinetics of phosphorylation and dephosphorylation of the major phosphoproteins appear to differ [10–12]. For example, the 9–10 kDa appears to be phosphorylated by a different kinase compared with LHC II polypeptides, since the phosphorylation of the latter is totally inhibited by the presence of the ADP analogue 5′-*p*-fluorosulphonylbenzoyl adenosine (FSBA) while that of the 9–10 kDa polypeptide is only partially inhibited [13]. Similarly, the amino-acid sequence of the phosphorylation site of 9–10 kDa and LHC II polypeptides differ and studies employing the sequence peptides of the LHC II phosphorylation site appear to be phosphorylated by a different protein kinase compared with the 9–10 kDa polypeptide [14].

Abbreviations: PS, Photosystem; LHC II, light-harvesting chlorophyll *a/b* binding protein complex II; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SDS, sodium dodecyl sulphate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll; FSBA, 5′-*p*-fluorosulphonylbenzoyl adenosine

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The phosphorylation of the LHC II polypeptides has been proposed to regulate the distribution of the excitation energy between the two photosystems [15]. The rate and the level of phosphorylation, and the kinetics of dephosphorylation, of the LHC II polypeptides have been reported to be closely correlated to fluorescence changes in the photosystems [10,11,16]. However, neither the rate and level of phosphorylation of the 9–10 kDa protein nor its kinetics of dephosphorylation exhibit a direct correlation with the fluorescence changes of the photosystems [10,11,17]. Other evidence has included the inhibitory effects of ADP or its analogs on the phosphorylation of LHC II and concomitant and parallel changes in the fluorescence parameters [13,17].

By contrast Markwell et al. [18] have shown that zinc ions inhibit the fluorescence decline but stimulate the phosphorylation of LHC II (although it has been suggested that the affect of the zinc ions may be consequence of charge neutralisation [13]). We have shown that there is little or no direct correlation between the time courses of the ATP-induced fluorescence decline and the rate and level of phosphorylation of LHC II polypeptides [12,19–21]. Moreover, little is known about the role of phosphorylation of the other proteins, many of which appear to reside in the appressed membrane zones with PS II and LHC II [3–6].

Almost all these studies have employed the adenine nucleotides or their analogs for determining the effect of phosphorylation on the fluorescence parameters. Several protein kinases are known to utilise ATP and GTP as substrates for phosphorylation [22–24] and different activities exhibit different substrate specificities. Millner et al. [25] have shown that the adenine nucleotides, e.g., ATP and ADP but not the guanine nucleotides, protect the kinase activity against the action of sulphydryl-directed agents by examining the variable fluorescence of PS II. They concluded that the binding of ATP or ADP to the kinase protects the sulphydryl groups on or close to the active site of the kinase, while the inactivity of the guanine nucleotides suggests that these nucleotides are not effective substrates. Regrettably they did not undertake direct studies, e.g., utilising labelled GTP, to substantiate this interpretation.

In this paper the phosphorylation of the LHC II polypeptides by GTP has been examined. It is shown that GTP can act as a substrate, though less efficiently than ATP, for the phosphorylation of several membrane proteins including LHC II. The time-course of LHC II phosphorylation and the fluorescence changes in the presence of ATP and GTP are also examined. The results are discussed with respect to the presence of different protein kinase activities, correlations between LHC II phosphorylation and the fluorescence decline and the possibility of an ATP binding site distinct from the substrate binding site of the protein kinase.

Materials and Methods

Freshly harvested spinach leaves were kept in the dark for up to 2 h at which time the leaves were homogenised and thylakoids were isolated as described in detail previously [12,19–21].

Thylakoid protein phosphorylation was initiated, in a reaction medium containing 30 mM Tricine (pH 8.0)/10 mM NaCl/5 mM MgCl/10 mM NaF/0.1 M sucrose and the NADPH-ferrodoxin reducing system as described previously [12,21], by adding the appropriate concentration of either ATP containing [γ-^{32}P]ATP or GTP containing [γ-^{32}P]GTP. Control thylakoids were similarly treated except that nucleotide was omitted from the reaction medium. The specific phosphate incorporation into the proteins, following fractionation by SDS-PAGE, and the fluorescence decline were determined as described in detail previously [12,19.26].

Autoradiography of phosphorylated proteins was achieved, after the fractionation of the proteins on 15% acrylamide gel by SDS-PAGE, by exposure of hyperfilm (Amersham) or X-Omat (Kodak) for 72 h. The autoradiograph was subsequently scanned at 500 nm in a Beckman DU-8 spectrophotometer equipped with a gel-scanning attachment.

All biochemicals were purchased from Sigma Chemical Co., reagents for SDS-PAGE from Bio-Rad. All other reagents were of Analar grade. High specific activity [γ-^{32}P]ATP (3000 Ci/mmol) and [γ-^{32}P]GTP (10 Ci/mmol) were purchased from Amersham International, Amersham.

Results

Isolated thylakoids were incubated in the presence of either 1 mM [γ-^{32}P]GTP or with 1 mM [γ-^{32}P]ATP and the phosphorylated proteins visualised by autoradiography, after fractionation by SDS-PAGE. The densitometric scans (Fig. 1) show that several polypeptides are labelled in the presence of the two nucleotides, including the LHC II polypeptides and the 9–10 kDa. The extent of phosphorylation by GTP is lower compared with ATP but more importantly at least two groups of polypeptides, co-migrating with apparent molecular weights of 36 kDa and 19 kDa, do not appear to be labelled by GTP (arrows in Fig. 1).

The K_m ATP and K_m GTP for the protein kinases phosphorylating the LHC II polypeptides and the 9–10 kDa protein were also determined. Isolated thylakoids were incubated in the presence of different concentrations of GTP or ATP containing the appropriate labelled nucleotide. The K_m for the nucleotides was determined by linear regression of Lineweaver-Burke plots. The K_m ATP was 40–60 μM and K_m GTP was 500–700 μM for the LHC II polypeptides while the V_{max} ATP was 0.48 and V_{max} GTP was 0.08 pm/min per μg chlorophyll

(Fig. 2). In the case of the 9–10 kDa the K_m ATP was 160–200 μM and K_m GTP was 200–300 μM (the K_m GTP and K_m ATP for the 50 kDa protein appeared to be similar to that for the LHC II polypeptides; data not shown).

The time-course of phosphorylation of the LHC II polypeptides by GTP and ATP was also examined. Isolated thylakoids were incubated in the presence of either 2 mM GTP or 1 mM ATP (Fig. 3). Under both the nucleotide regimes there is rapid incorporation into the LHC II polypeptides with no apparent lag-phase

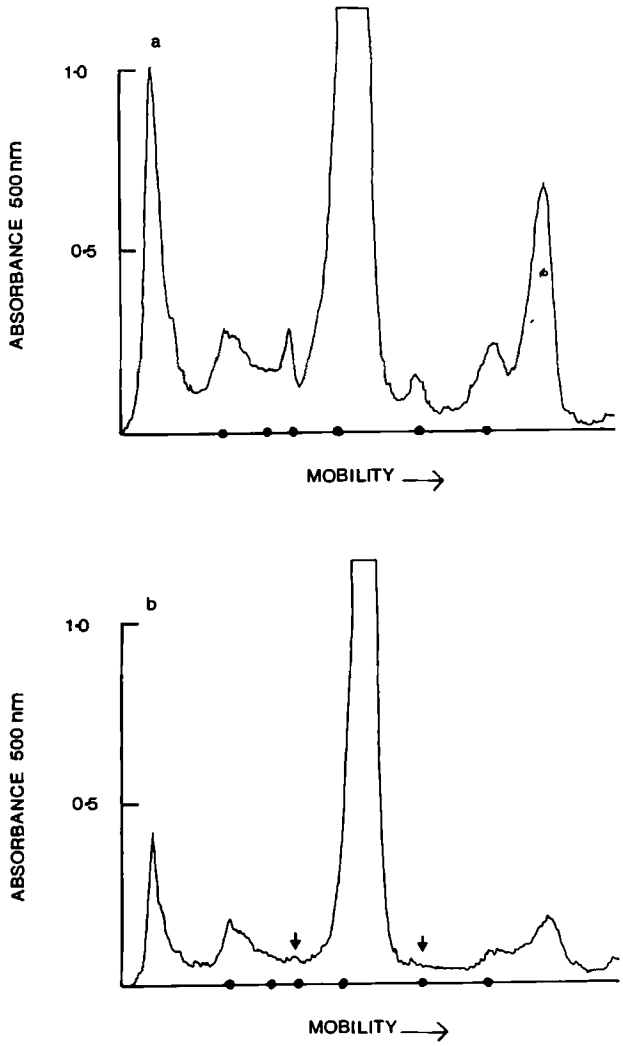

Fig. 1. Densitometric scans of autoradiographs of γ-^{32}P-labelled thylakoid membrane proteins incubated in the presence of either labelled (a) ATP or (b) GTP. Isolated thylakoids were phosphorylated by addition of either 1 mM ATP or 1 mM GTP for 60 min at which time the reaction was terminated by the addition of ice-cold acetone and the precipitated proteins fractionated by SDS-PAGE. A hyperfilm sheet was exposed to the dried gel for 72 h, developed and scanned at 500 nm using a Beckman DU-8 spectrophotometer containing a gel scanning attachment. The arrows indicate the proteins which contain co-migrating γ-^{32}P label when incubated in the presence of [γ-^{32}P]ATP but remain unlabelled in the presence of [γ-^{32}P]GTP. Molecular weight markers indicated on the mobility axis are: 66000, 45000, 36000, 29000, 20100 and 14000, respectively.

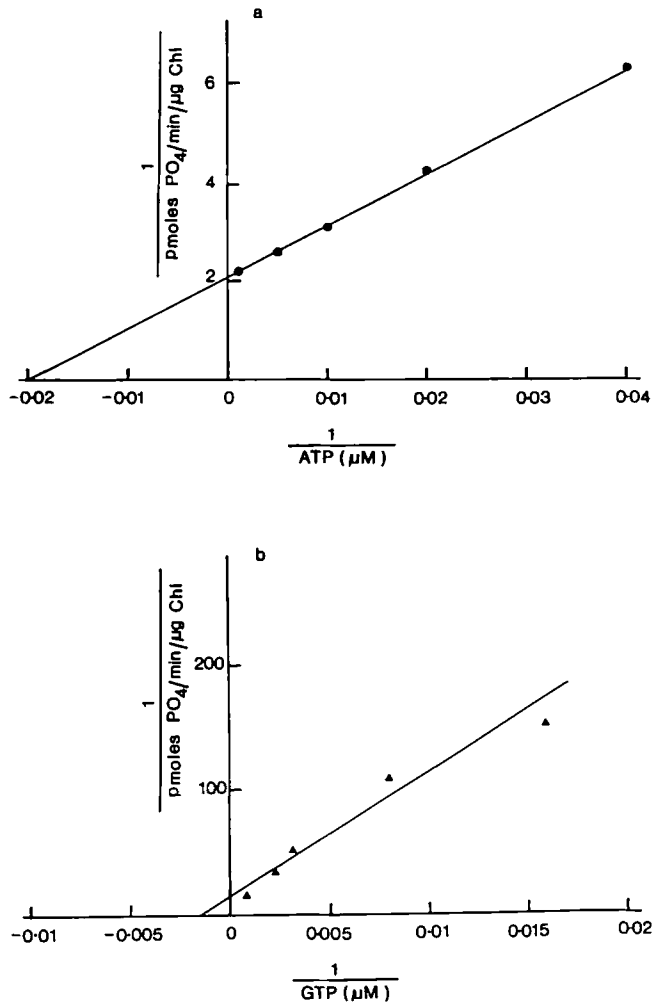

Fig. 2. Isolated thylakoids were incubated in the presence of varying concentrations of (a) ATP or (b) GTP for 2 min. The reaction was then terminated and the proteins fractionated by SDS-PAGE. LHC II polypeptides were excised from the gel and the co-migrating label determined by liquid scintillation. The K_m for ATP and GTP are 40–50 μM or 500–700 μM, respectively.

observed and phosphorylation continues for up to 2 h. Both the 23 kDa and the 25 kDa LHC II polypeptides are phosphorylated in the presence of GTP, in a manner similar to that observed with ATP [12,21]. Typically the initial rate of phosphorylation of the 23 kDa was about 3–4-fold faster (four different experiments) compared with the 25 kDa polypeptide. However, the phosphate incorporation in the presence of ATP is about 6-fold faster compared with GTP but tends to deviate from linearity much earlier, achieving a plateau by 90 min. The non-linearity observed with ATP is most probably a consequence of substrate limitation by LHC II [21] and GTP achieves up to 50–60% phosphorylation when compared with ATP (not shown).

Incubation of isolated thylakoids in the presence of 1 mM unlabelled GTP (2 h) followed by the addition of 100 μM labelled ATP exhibits only 50% phosphate incorporation compared with a control sample (in-

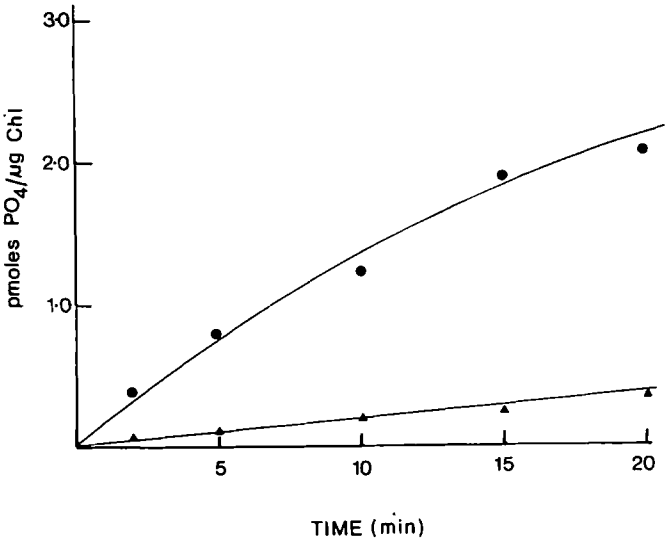

Fig. 3. The time-course of phosphorylation of the LHC II polypeptides in the presence of ATP (●——●) or GTP (▲——▲). Isolated thylakoids were incubated for the indicated times in the presence of either 1 mM ATP or 2 mM GTP containing the appropriate labelled nucleotide. The reaction was then terminated and the specific incorporation into the LHC II polypeptides determined as described in Materials and Methods.

cubated in the absence of GTP and similarly challenged). Under the same conditions after pre-incubation with unlabelled ATP less than 5% incorporation is observed compared to the control sample. The observed decrease in phosphorylation would be consistent with the amount of LHC II protein pre-phosphorylated by GTP (about 50%) and ATP (over 95%). The LHC II polypeptides are known to be phosphorylated on a threonine residue [27] and the inhibition by GTP of the ATP-phosphorylation would indicate that the same sites are phosphorylated by both nucleotides.

ATP-induced phosphorylation has been reported to lead to a decline in the room temperature fluorescence of PS II [3–6]. I have more recently shown that even at sub-optimal ATP concentration (the rate of phosphorylation was more than 4-fold slower compared with 1 mM ATP) a fluorescence decline is observed [28]. It was therefore of interest to examine whether the GTP-induced phosphorylation, the phosphorylation rate is similar to that observed with sub-optimal ATP (Figs. 2 and 3 and Ref. 28), also induced a fluorescence decline. After various times of incubation either in the presence of ATP or GTP or in the absence of nucleotide (control) thylakoids were removed and diluted 200-fold with ice-cold buffer and the fluorescence quenching determined [12,19–21]. While a fluorescence decline is observed with ATP, achieving a stable plateau between 5–20 min (see also Refs. 12 and 19–21), little or no fluorescence decline was observed during the experimental period in the presence of GTP. Indeed after 15–20 min of GTP-induced phosphorylation the level of phosphorylation of the LHC II polypeptides is close to that observed after 2 min with ATP.

The addition of EDTA (4 mM) to chelate the magnesium ions and thereby lower the concentration of the screening cations [19] increased the fluorescence decline of thylakoids pre-phosphorylated in the presence of ATP by about 2-fold but exhibit no change in the case of thylakoids pre-phosphorylated by GTP. In contrast, the addition of 100 μM ATP to thylakoids incubated with 1 mM GTP leads to an almost complete fluorescence decline being observed (not shown), suggesting that GTP does not inhibit the fluorescence decline observed with ATP (see also Ref. 25).

Discussion

In recent years a great deal of attention has been directed on the phosphorylation of LHC II proteins, since it has been proposed that phosphorylation of these proteins may control the redistribution of excitation energy between the photosystems (see Introduction). A close correlation between the rate and extent of LHC II phosphorylation as well as kinetics of dephosphorylation and the fluorescence changes of the photosystems has been reported [10,11,16]. These data inherently imply a causative relationship between these two phenomena. Similarly, the ADP analogs such as FSBA also suggest that LHC II phosphorylation is implicated in the fluorescence change [13]. However, in these studies [10,11,13,16,17] the phosphorylation of other proteins, and most notably the 9–10 kDa protein, are clearly shown not to be correlated with the accompanying fluorescence changes. In contrast, other studies [12,18–21,28] suggest no direct correlation between the rate and extent of phosphorylation of LHC II polypeptides and the fluorescence changes.

Furthermore, Thornber and co-workers have shown that the adenylate charge can regulate the fluorescence changes, possibly by altering the protein kinase activity [29]. In this context it is interesting to note that Millner et al. [25] have shown that blocking the sulphydryl groups of the kinase leads to deactivation of the protein kinase activity and affects the variable fluorescence of PS II. However, pre-incubation with either ATP or ADP, but not with other nucleotides, e.g., GTP, protects against deactivation of the protein kinase activity.

In common with other protein kinases [22–24] at least some of the endogenous protein kinases present in thylakoid membranes can utilise both ATP and GTP as substrates for phosphorylation (Figs. 1 and 2). Similar to the chick brain protein kinase [22] the efficiency of phosphorylation by GTP is significantly less compared with ATP (Figs. 2 and 3). However, there are at least two groups of proteins which do not appear to undergo GTP-induced phosphorylation (Fig. 1), suggesting that

they utilise an ATP-dependent protein kinase in contrast to the LHC II and the 9–10 kDa proteins.

Previous studies have implied that the 9–10 kDa protein is phosphorylated by a different protein kinase compared with the LHC II protein [10,11,13,14]. Both these kinase activities can though utilise either ATP or GTP as substrates (Figs. 1 and 2) and the observation that the K_m ATP and K_m GTP for the protein kinase phosphorylating the 9–10 kDa protein are similar but that the K_m ATP and K_m GTP for the protein kinase phosphorylating the LHC II protein are significantly different would support the notion that two different protein kinases phosphorylate these two major phosphoproteins. A third protein kinase activity which is totally ATP-dependent phosphorylates the two groups of polypeptides, 36 kDa and 19 kDa, co-migrating just before and after the LHC II polypeptides (see Fig. 1).

As GTP can act as a substrate for the protein kinase which phosphorylates the LHC II protein it must therefore bind to the active site of the protein kinase. While the data of Millner et al. [25] suggests that guanine nucleotides do not protect against the action of the sulphydryl-directed reagents. Clearly as GTP acts as a substrate (Figs. 1–3) the protection afforded by the adenine nucleotides cannot be a consequence of binding to the active site on the protein kinase. An alternative interpretation would be that there is another distinct site to which the ATP or ADP binds to protect against these reagents. If the SH groups implicated in this interaction are close or in the active site of the protein kinase [25] then the binding of ATP or ADP to this second site must cause a conformational change in the kinase or in the membrane. Furthermore, as this action cannot be brought about by the guanine nucleotides this second site must therefore be specific for the adenine nucleotide. It is therefore, at least theoretically, possible that the adenylate charge can affect this site in vivo.

Finally, it has been shown that GTP leads to the phosphorylation of the LHC II protein. LHC II on the isolated thylakoids is known to be phosphorylated on the threonine residue of the trypsin-cleavable segment [27]. The same site is presumably phosphorylated by ATP and GTP, as GTP-induced phosphorylation results in a decrease in the phosphorylation of LHC II by ATP compared with the control (data not shown). However, unlike ATP-induced phosphorylation the GTP-induced phosphorylation of the LHC II protein does not result in a fluorescence decline (Fig. 4) even when the concentration of the screening cations is sub-saturating. It can be argued that the level of phosphorylation with GTP is too low to induce the fluorescence decline. However, in this context it should be mentioned that we have failed to find any direct correlation between the rate and level of phosphorylation of LHC II and the fluorescence decline [12,19–21,28,30]. More importantly at sub-optimal concentrations of ATP the rate and level

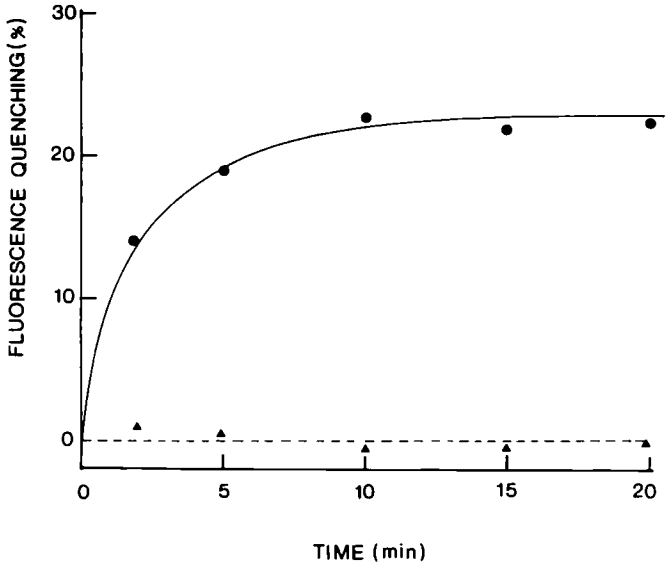

Fig. 4. The time-course of the Photosystem II fluorescence decline of thylakoids incubated in the presence of either ATP (●———●) or GTP (▲- - - - -▲). Isolated thylakoids were incubated under identical conditions as in Fig. 3. At various times aliquots were removed and the reaction terminated by the addition of 200-fold ice-cold phosphorylation medium. The fluorescence quenching was determined as described in Materials and Methods.

of phosphorylation of LHC II is only slightly faster than that observed with GTP (4-fold compared with 6-fold slower than that observed with optimal ATP concentrations [28]), yet the time-course of the fluorescence decline is similar to that observed at the higher ATP concentration.

These data raise important questions about the validity of the reported correlations between LHC II phosphorylation and the fluorescence decline [10,11,16]. For example it may be that the phosphorylation of some other thylakoid proteins is essential, and at least 20 polypeptides have been shown to be phosphorylated both in vivo and in vitro [1,2]. In the case of GTP-induced phosphorylation at least two groups of polypeptides are not phosphorylated (Fig. 1) and no fluorescence decline is observed (Fig. 4.). Alternatively it can be argued that ATP itself may in some manner cause a conformational change in the membrane [28,30], a change not brought about by GTP, which can lead to a fluorescence decline perhaps in a co-operative manner with LHC II phosphorylation or that of other proteins.

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References

1 Suss, K.H. (1981) Biochem. Biophys. Res. Commun. 102, 724–728.
2 Bennett, J. (1977) Nature (London) 269, 344–346.
3 Bennett, J. (1983) Biochem. J. 212, 1–13.
4 Barber, J. (1986) in Encyclopedia of Plant Physiology 19, Photosynthesis 19, Photosynthesis III (Staehlin, L.A. and Arntzen, C.J., eds.), pp. 653–665, Springer-Verlag, Berlin.
5 Anderson, J.M. (1986) Ann. Rev. Plant Physiol. 37, 93–136.
6 Murphy, D.J. (1987) Biochim. Biophys. Acta 864, 33–94.
7 Lin, Z.-F., Lucero, H.A. and Racker, E. (1982) J. Biol. Chem. 257, 12153–12157.
8 Lucero, H.A., Lin, Z.-F. and Racker, E. (1982) J. Biol. Chem. 12157–12160.
9 Bennett, J., Shaw, E.K. and Bakr, S. (1987) FEBS Lett. 210, 22–26.
10 Black, M.T., Foyer and Horton, P. (1984) Biochim. Biophys. Acta 767, 557–562.
11 Steinback, K.E., Bose, S. and Kyle, D.J. (1982) Arch. Biochem. Biophys. 216, 356–361.
12 Islam, K. and Jennings, R.C. (1985) Biochim. Biophys. Acta 810, 158–163.
13 Frachaus, J., Dilley, R.A. and Cramer, W.A. (1985) Biochim. Biophys. Acta 809, 17–26.
14 Michel, H.P. and Bennett, J. (1987) FEBS Lett. 212, 103–108.
15 Allen, J.F., Bennett, J., Steinback, K.E. and Arntzen, C.J. (1981) Nature (London) 291, 25–29.
16 Horton, P., Allen, J.F., Black, M.T. and Bennett, J. (1981) FEBS Lett. 125, 193–196.
17 Baker, N.R., Markwell, J.P. and Thornber, J.P. (1982) Phobiochem. Photobiophys. 4, 211–217.
18 Markwell, J.P., Baker, N.R., Bradbury, M. and Thornber, J.P. (1984) Plant Physiol. 74, 348–354.
19 Jennings, R.C., Islam, K. and Zucchelli, G. (1986) Biochim. Biophys. Acta 850, 483–489.
20 Zucchelli, G., Islam, K. and Jennings, R.C. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. II, pp. 725–728, Martinus Nijhoff, Dordrecht.
21 Islam, K. (1987) Biochim. Biophys. Acta 893, 333–341.
22 Burns, R.G. and Islam, K. (1984) Biochem. J. 224, 623–627.
23 Piras, M.M. and Piras, R. (1974) Eur. J. Biochem. 47, 443–452.
24 Edelman, A.M., Blumenthal, D.K. and Krebs, E.G. (1987) Ann. Rev. Biochem. 56, 567–613.
25 Millner, P.A., Widger, W.R., Abbott, M.S., Cramer, W.A. and Dilley, R.A. (1982) J. Biol. Chem. 257, 1736–1742.
26 Torti, F., Gerola, P.D. and Jennings, R.C. (1984) Biochim. Biophys. Acta 767, 321–325.
27 Mullett, J.E., Baldwin, T.O. and Arntzen, C.J. (1981) in Photosynthesis III, Structure and Molecular Organisation of the Photosynthetic Membrane (Akoyunoglou, G., ed.), pp. 571–582, Balaban International Science Services, Philadelphia, PA.
28 Islam, K. (1987) Biochem. Soc. Trans. 16, 46–47.
29 Markwell, J.P., Baker, N.R. and Thornber, J.P. (1982) FEBS Lett. 142, 171–174.
30 Islam, K. (1989) Biochim. Biophys. Acta 974, 267–273 (subsequent article)