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Relative kinetics of quenching of Photosystem II fluorescence and phosphorylation of the two light-harvesting chlorophyll *a/b* polypeptides in isolated spinach thylakoids

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The kinetics of phosphorylation of the two light-harvesting chlorophyll *a/b* polypeptides in the presence of ATP, reduced plastoquinone and 5 mM Mg^{2+} shows increasing phosphorylation with time. The kinetics of quenching of the Photosystem II fluorescence at 5 mM Mg^{2+} does not parallel the observed kinetics of phosphorylation, but achieves a steady level of quenching after the first few minutes. By contrast, the fluorescence parameters of thylakoids phosphorylated in the presence of 5 mM Mg^{2+} and subsequently transferred to 2 mM Mg^{2+} exhibit a continuing decline in the phosphorylation-induced quenching and a decrease in the F_m/F_0 ratio compared with the non-phosphorylated controls. The phosphorylation-induced quenching at the magnesium concentrations is discussed in terms of detachment of the light-harvesting chlorophyll *a/b* polypeptides from the Photosystem II matrix and 'spillover' type mechanisms.

Introduction

The thylakoid membranes of green plants contain protein kinase(s) and phosphatase(s) which reversibly phosphorylate a number of proteins in vivo and in vitro, including the most conspicuous light-harvesting chlorophyll *a/b* protein (for reviews, see Refs. 1–3). Activation of the kinase(s) requires the reduction of the plastoquinone pool, ATP and magnesium ions [4–7] and membrane phosphorylation causes a decrease in the room temperature fluorescence emission of between 15–20% [8,9]. The phosphorylation of the LHCP and its regulation by the redox state of the plastoquinone has been proposed to constitute a molecular mechanism for the controlled redistribution of excitation energy between the two photosystems PS I and PS II [10]. It is generally agreed that a fraction of the LHCP becomes detached from the PS II-LHCP matrix in the phosphorylated membranes and subsequently associates and transfers energy to PS I [3,11,12]. It should be mentioned that this relatively simple picture pertains to chloroplasts incubated in the presence of saturating magnesium concentrations. At lower magnesium concentrations the ATP-induced fluorescence decline has been reported to be much greater than 15–20% [2] and in addition to LHCP movement from PS II to PS I, there appears to be an additional quenching phenomenon, possibly due

Abbreviations: LHCP, light-harvesting chlorophyll *a/b* protein; PS, Photosystem; F_0, non-variable fluorescence; F_m, maximal fluorescence in the presence of DCMU; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; F_mP and F_mNP, fluorescence yield of the phosphorylated and nonphosphorylated thylakoid membranes, respectively.

to a 'spill-over' type interaction between PS II and PS I [13,14].

LHCP is compsoed of at least two polypeptides [15–18], and in spinach consists of a major and a minor polypeptide of 25000 M_r and 23500 M_r, respectively, both of which can be phosphorylated [18]. Studies on the ATP-induced fluorescence decline and the amount of γ-^{32}P incorporation into the LHCP polypeptides suggest an almost perfect correlation between these two parameters [6]. More recently, Black et al. [19] have reported a close correlation between the rate of phosphorylation of LHCP and the ATP-induced fluorescence decline. Similarly the kinetics of dephosphorylation closely parallel a decrease in the 77 K fluorescence emission at 735 nm relative to that at 685 nm (F_{735}/F_{685}), as well as a decrease in the fluorescence quenching at room temperature [20]. By contrast, Markwell et al. [21] have reported that under conditions when Zn^{2+} stimulates the phosphorylation of LHCP both the ATP-induced PS II fluorescence decline and the increase in the F_{735}/F_{685} are inhibited.

In this paper we examine the kinetics of phosphorylation of the two LHCP polypeptides and the ATP-induced PS II fluorescence decline in thylakoids incubated at two different Mg^{2+} concentrations. We tentatively conclude that in fully stacked chloroplasts incubated at saturating Mg^{2+} concentrations only a fraction of the phosphorylated LHCP becomes detached from the PS II-LHCP matrix.

Materials and Methods

Young freshly harvested spinach leaves were kept in the dark at room temperature to allow protein dephosphorylation by endogenous phosphatase(s). Isolated thylakoids were then prepared as described in detail previously [12], except that the magnesium ion concentration was adjusted to 5 mM, and used immediately for the experiments. The thylakoids were allowed to equilibrate for 3–4 min at room temperature in the phosphorylation medium consisting of 30 mM Tricine (pH 8.0)/10 mM NaCl/5 mM $MgCl_2$/10 mM NaF/0.1 M sucrose in the presence of the NADPH-ferrodoxin reducing system (the plastoquinone pool is 80–90% reduced under these conditions [12]). Phosphorylation was initiated by the addition of 1 mM Mg-ATP and at various times aliquots were removed and the reaction terminated either by the addition of 100-fold excess of ice-cold phosphorylation medium at the desired Mg^{2+} concentration for determination of the fluorescence parameters or by addition of ice-cold 80% acetone to precipitate the proteins for analysis by SDS-polyacrylamide gel electrophoresis. Control samples were treated in an identical manner, except in the absence of ATP.

Following dilution the thylakoids were kept on ice for about 30 min before the determination of the fluorescence parameters (F_m and F_0) [22]. Acetone-precipitated proteins were resuspended in a solution containing 45 mM Na_2CO_3/5% 2-mercaptoethanol/2.5% lithium dodecyl sulphate/15% sucrose/0.5% bromophenol blue. The samples were heated at 70°C for 5 min and electrophoresed on a 10–20% SDS-polyacrylamide gradient gel, stained with 0.025% Coomassie brilliant blue R-250/10% acetic acid/25% isopropan-1-ol and destained in 10% acetic acid [23]. The gel was dried and autoradiographed using a Kodak X-Omat X-ray film. γ-^{32}P in the excised gel bands was determined by counting in a Packard Tricarb liquid scintillation spectrometer in the Packard Insta-Gel liquid scintillant.

Ferrodoxin was prepared by the method of Ashton and Anderson [24], and LHCP purified by the procedure of Ryrie et al. [17].

All biochemicals were purchased from Sigma Chemical Co., reagents for SDS-polyacrylamide gel electrophoresis from Bio-Rad. All other reagents were of Analar grade. High specific activity [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from the Radiochemical Centre, Amersham.

Results

When isolated chloroplast thylakoids were incubated with [γ-^{32}P]ATP, following the chemical activation of the protein kinase(s), rapid incorporation of γ-^{32}P label into several membrane proteins was observed. The results of a typical experiment are shown in Fig. 1. The most prominently labelled proteins have apparent molecular weights of 26, 24 and 9–11 kDa, although with time a number of other proteins also incorporate signifi-

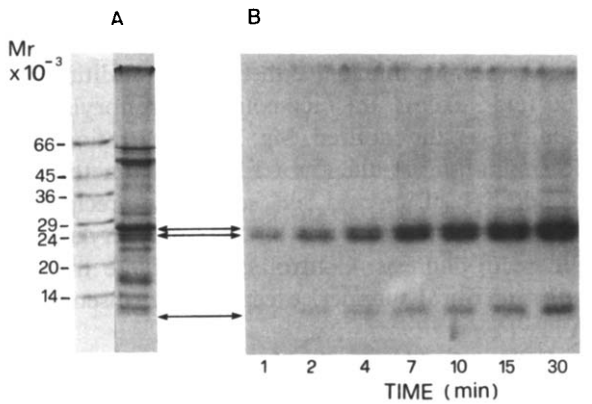

Fig. 1. Phosphorylation of isolated thylakoid membrane proteins by endogenous protein kinase(s). Isolated thylakoids (240 μg chlorophyll/ml) were incubated in the presence of 1 mM Mg-ATP and at the indicated times 100 μl aliquots were withdrawn and the reaction terminated by the addition of 80% ice-cold acetone. The precipitated proteins were fractionated by SDS-polyacrylamide electrophoresis (A) and the phosphorylated proteins visualised by autoradiography (B). The position of the 26 and 24 kDa LHCP polypeptides, identified by comigration with purified LHCP, and the 9–11 kDa protein are indicated. The molecular weight standards indicated are: bovine serum albumin (66000), ovalbumin (45000), glyceraldehyde 3-phosphate dehydrogenase (36000). carbonic anhydrase (29000), trypsinogen (24000), trypsin inhibitor (20100), lactalbumin (14200).

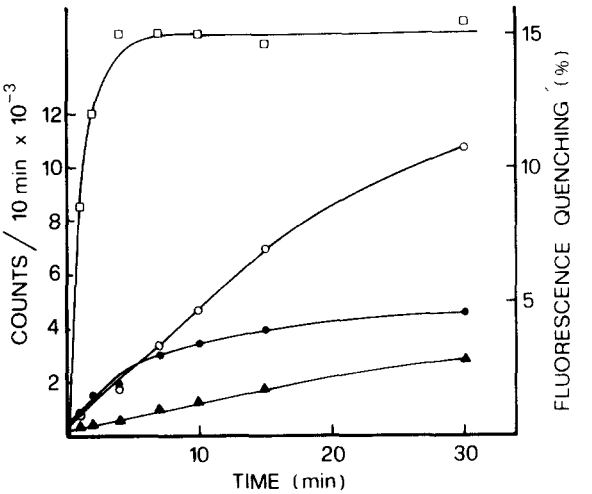

Fig. 2. Time-course of γ-^{32}P incorporation into specific proteins and of the fluorescence quenching. The γ-^{32}P label comigrating with the low- (●) and high-molecular-weight LHCP (○), and the 9–11 kDa protein (▲), was determined by liquid scintillation, following fractionation on SDS-polyacrylamide gel electrophoresis and excision of the protein band from the gel (see Fig. 1), and is expressed as counts per 10 min of the excised gel band. The fluorescence yield of the phosphorylated (F_mP) and the non-phosphorylated (F_mNP) thylakoid membranes was determined following induction with DCMU. The fluorescence quenching (□), defined as $(F_m\text{NP} - F_m\text{P})/F_m\text{NP}$, is expressed as a percentage of the control F_m.

cant label (Fig. 1B). The major and minor Coomassie staining protein bands of M_r 26000 and 24000 (Fig. 1A) were identified as the LHCP polypeptides, by co-electrophoresis with the LHCP purified by the procedure of Ryrie et al. [17]. A small amount of unidentified material which failed to enter the gel was also labelled as reported previously by other workers [20,25].

A qualitative assessment of the autoradiograph suggests that most of the γ-^{32}P label incorporated into the thylakoid proteins co-migrates with the two LHCP polypeptides. However, the lower-molecular-weight LHCP appears to be phosphorylated much more rapidly compared with the higher-molecular-weight LHCP.

The apparent differences in the rate of phosphorylation of the proteins were therefore further examined. The time-course of γ-^{32}P incorporation into the higher- and lower-molecular-weight LHCP and the 9–11 kDa proteins was determined (Fig. 2). There was no apparent lag-period and increasing γ-^{32}P label was incorporated into each of these proteins with time, although maximal phosphorylation was not attained even after 30 min. However, the kinetics of the low-molecular-weight LHCP differ markedly from those of the high-molecular-weight LHCP and the 9–11 kDa proteins. Thus while the rate of phosphorylation of the latter proteins was approximately linear for up to 15 min, that of the low-molecular-weight LHCP clearly deviated from linearity after 5–6 min. A quantitatively similar pattern of phosphorylation was also noted when light rather than the chemical procedure was employed to activate the kinase(s) (data not shown), confirming that the two procedures effect phosphorylation in a similar manner.

The fluorescence yield of the phosphorylated (F_mP) and the nonphosphorylated (F_mNP) thylakoid membranes was also determined as a function of time in the same experiment (Fig. 2). The ATP-induced PS II fluorescence quenching (determined as $(F_m\text{NP} - F_m\text{P})/F_m\text{NP} \times 100$) in-

creased initially in the first 4–5 min, after which a more or less steady level was attained. Little or no further increase was observed in the 5–30 min period, yet substantial γ-^{32}P label was incorporated into the LHCP and other proteins over the same time-period (Fig. 1 and 2). We have noticed variability in the kinetics of the fluorescence decline, and in the γ-^{32}P incorporation between experiments. However, the relationship depicted in Fig. 2, whereby substantial γ-^{32}P incorporation into LHCP occurs with little or no corresponding change in the fluorescence quenching, has always been observed.

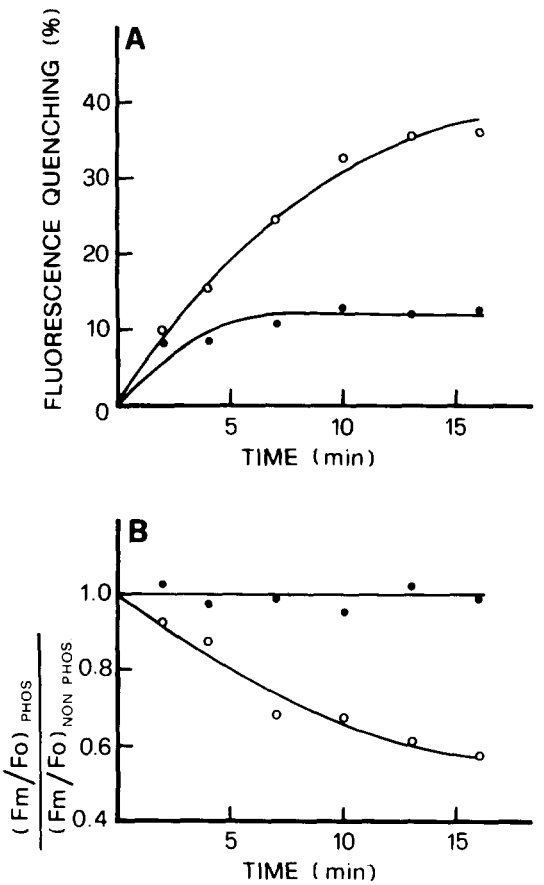

Fig. 3. Time-course of phosphorylation-dependent changes in fluorescence parameters at two different magnesium ion concentrations. Isolated thylakoids (235 μg chlorophyll/ml) were incubated with 1 mM Mg-ATP in the phosphorylation medium containing 5 mM Mg^{2+} (see Materials and Methods). At various times aliquots were removed and suspended in 100-fold excess of the phosphorylation medium containing either 5 mM Mg^{2+} (●) or 2 mM Mg^{2+} (○) and the F_0 and the F_m values were determined. (A) Fluorescence quenching percentage and (B) comparison of the F_m/F_0 ratio of the phosphorylated/non-phosphorylated thylakoids.

The effect of varying the cation concentration on fluorescence parameters subsequent to protein phosphorylation was also examined. Protein phosphorylation was initiated by the addition of ATP in the phosphorylation medium containing 5 mM Mg^{2+}. After various times of phosphorylation aliquots were removed and suspended in cold phosphorylation medium containing either a saturating cation concentration (5 mM Mg^{2+}) or a slightly subsaturating cation concentration (2 mM Mg^{2+}) as described in Materials and Methods. The time-course of the phosphorylation-induced changes in the F_m and the F_0 values were determined (Fig. 3). As already noted in Fig. 2, an initial decrease in the F_m value of the phosphorylated thylakoids was observed compared with the non-phosphorylated control, followed by the attainment of a steady-level of fluorescence quenching in the presence of the saturating cation concentration. By contrast thylakoids suspended in the presence of the subsaturating cation concentration showed a continued decline in the F_m over the entire experimental period. Differences were also observed with respect to the F_m/F_0 ratio for thylakoids suspended at these two cation concentrations as a consequence of phosphorylation. While the F_m/F_0 ratio remained unaltered with increasing time of phosphorylation in the case of thylakoids suspended at 5 mM Mg^{2+}, it continued to decline for those suspended at 2 mM Mg^{2+}.

Discussion

The phosphorylation of thylakoid proteins has been implicated in the control of energy distribution between PS I and PS II (see Introduction). A number of proteins is phosphorylated in the presence of ATP (Fig. 1), and in agreement with numerous previous reports the LHCP and the 9–11 kDa proteins are the major substrates for the endogenous protein kinase(s) (for example, see Refs. 1–3). However, the two LHCP polypeptides have been reported to be labelled with either the same specific activity [26] or with differing specific activities [11,18]. In agreement with the latter report we also observe a higher specific activity of labelling of the low-molecular-weight LHCP, since

it constitutes only a minor protein band (Figs. 1 and 2).

The time-course of phosphorylation of LHCP and the 9–11 kDa proteins shows significant differences in the kinetics of phosphorylation of the low-molecular-weight LHCP compared with those observed for the high-molecular-weight LHCP and the 9–11 kDa proteins (Fig. 1). It is perhaps worth mentioning that we have noted quantitatively similar results when light was used to activate the protein kinase(s). The differences in the kinetics of these proteins may be interpreted in terms of different protein kinase activities, as multiple protein kinases have been reported in spinach [27]. Alternatively, as suggested by Black et al. [19], a single protein kinase activity may account for the phosphorylation of all these proteins. In this case the observed differences in the phosphorylation kinetics may reflect the properties of the low-molecular-weight LHCP as a substrate for phosphorylation.

LHCP has been reported to detach from the PS II-LHCP matrix upon phosphorylation [3,11,12] and a close correlation between phosphorylation of LHCP and the ATP-induced PS II fluorescence decline has also been observed [2,6,19]. These observations have led to the widely held convinction that it is the phosphorylation of LHCP itself which mediates its movement away from PS II, although a recent report has questioned this causative relationship [21]. Electrostatic double-layer phenomena are also thought to be involved [28–30].

We show that substantial phosphorylation occurs after the first 5 min when the fluorescence quenching achieves a steady level, in thylakoids suspended in the presence of 5 mM Mg^{2+} (Fig. 2). By contrast Steinback et al. [20] and Black et al. [19] have reported a correlation between LHCP phosphorylation and fluorescence quenching. However, it should be pointed out that either the kinetics of dephosphorylation were examined [20] or the initial rate of phosphorylation was determined at varying ATP concentrations [19]. The different results may well be due to the different experimental strategies employed.

The 5 mM Mg^{2+} concentration used in these experiments is saturating with respect to both the electrostatic screening requirement for thylakoid appression and fluorescence changes, and the effects of thylakoid phosphorylation on various PS II fluorescence parameters. We would therefore tentatively suggest that in the presence of saturating screening cations only a fraction of the phosphorylated LHCP becomes detached from the PS II-LHCP matrix. This may be due to steric or structural factors, such that for example only LHCP which is located peripherally in the PS II-LHCP matrix has the potential to detach and move towards the PS I-enriched membrane zones, though other hypotheses are also tenable.

In contrast to the situation of 5 mM Mg^{2+}, thylakoids incubated in the presence of subsaturating cation concentrations exhibit a continued decline in F_m with increasing time of phosphorylation (Fig. 3A). Similarly, the F_m/F_0 ratio at the subsaturating cation concentration continues to decrease, unlike that observed at the saturating cation concentration (Fig. 3B). This large phosphorylation-induced quenching observed at the subsaturating concentrations of Mg^{2+} suggests that LHCP detachment and quenching by PS I occur together with a direct interaction between PS I and PS II of the 'spillover' type [13,14,31]. The decline observed in the F_m/F_0 ratio is in agreement with such an interpretation.

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