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AN INVESTIGATION INTO THE ATP REQUIREMENT FOR PHOSPHORYLATION OF THYLAKOID PROTEINS AND FOR THE ATP-INDUCED DECREASE IN THE YIELD OF CHLOROPHYLL FLUORESCENCE IN CHLOROPLASTS AT DIFFERENT STAGES OF DEVELOPMENT

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The phosphorylation of thylakoid membrane polypeptides has been investigated in chloroplasts prepared from peas that had been grown under intermittent light and then exposed to between 4 and 48 h of continuous light. At 4 h, when the ratio of the total amount of labelling of a 9 kDa-polypeptide relative to light-harvesting chlorophyll protein (LHCP) polypeptides was much greater than 1, the affinity for ATP was found to be the same ($S_{0.5}$, approx. 100 μM) for both polypeptides. In contrast, in fully greened chloroplasts, when labelling of LHCP was much greater than that of the 9 kDa-polypeptide, the $S_{0.5}$ for ATP was 40 μM for LHCP and 500 μM for the 9 kDa-polypeptide. A correlation was observed during development between the affinity for ATP of the 9 kDa-species and its abundance relative to LHCP. It is suggested that these polypeptides compete for phosphorylation by the same protein kinase. Simultaneous assay of the ATP-induced fluorescence decrease at different ATP concentrations revealed a close correlation with LHCP labelling but not with labelling of the 9 kDa-polypeptide. This correlation held irrespective of which polypeptide was the major phosphoprotein.

Introduction

The protein kinase of chloroplast thylakoids catalyses the phosphorylation of a number of membrane proteins (for reviews see Refs. 1 and 2). In terms of the amount of ^{32}P incorporation into thylakoid protein, the major phosphoprotein is the light-harvesting complex of PS II (LHCP) but there are several other phosphorylated polypeptides which also separate with PS II upon detergent fractionation of thylakoids [3]. The principle one of these has an apparent molecular weight of 9000.

In experiments in which the activity of the protein kinase is varied by means of illumination time [4], illumination quality [5], electron-transfer pathway [6] or redox potential [7], the 9 kDa- and LHCP polypeptides changed their phosphorylation level in parallel. These observations suggest that a single kinase population catalyses thylakoid protein phosphorylation. However, the use of Zn^{2+} and some inhibitory treatments of thylakoids led to the conclusion that there are several different thylakoid protein kinases [8,9], and that these kinases have either a low k_m for ATP (less than 0.2 mM) or a high k_m for ATP (more than 0.2 mM). Isolation of proteins with kinase activity from

Abbreviations: LHCP, Light-harvesting chlorophyll protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PS I, II, Photosystem I, II.

chloroplasts has shown the existence of at least two kinase types [10]. It has also been shown that phosphopolypeptides such as LHCP and the 9 kDa-species have different dephosphorylation kinetics; thus the [Mg^{2+}] requirement is higher [11] and the reaction rate slower [3,11] for the dephosphorylation of the 9 kDa polypeptide relative to LHCP.

A major function of protein phosphorylation is to alter the distribution of excitation between PS II and PS I [1,2], an effect which is probably brought about by detachment of LHCP from PS II [14] and its migration to the PS I-enriched unappressed membranes [15]. The decrease in the level of chlorophyll fluorescence excitation that results from phosphorylation was inferred to result from this change in distribution of excitation [12–14]. However, in view of the fact that several factors can alter the yield of chlorophyll fluorescence, the possibility remains that the observed effects of phosphorylation are not solely due to phosphorylation of LHCP and that direct effects on PS II are involved. Recently, for example, the causative relationship between LHCP phosphorylation and the fluorescence changes has been questioned [8]. In fact, only a single report has focussed directly on the question as to which polypeptide brings about the fluorescence change as a result of its phosphorylation; this utilized the above-mentioned difference in dephosphorylation kinetics and suggested that only LHCP phosphorylation was responsible for the fluorescence changes [3].

The work described in this paper addresses itself to two important questions concerning protein phosphorylation; firstly, that of the existence of different kinases for different protein substrates and secondly, that of the relationship between LHCP phosphorylation and fluorescence changes. A detailed examination of the ATP requirement for phosphorylation of the two major phosphoproteins and for the ATP-dependent decrease in chlorophyll fluorescence was carried out. This examination involved the use of both mature and partially greened intermittent-light chloroplasts which show vastly different phosphoprotein populations.

Materials and Methods

Peas (*Pisum sativum* var. Kelvedon Wonder) were grown for approx. 14 days in a growth chamber under a 16 h photoperiod. Intermittent-light peas were grown for 8–10 days in complete darkness and subsequently transferred to a flash chamber [16] and flashed for 48 h on a cycle of 118 min dark in each 2 h. Plants were then subjected to 4 h continuous light before chloroplast isolation. After a 4-h greening period they have a chlorophyll *a*/*b* ratio of over 5 and relatively little LHCP which is reflected in the largely exponential room-temperature fluorescence induction curve. In each case thylakoids were isolated as in Ref. 17.

Intermittent-light and mature thylakoids were incubated for 5 min in reaction medium containing 0.33 M sorbitol/10 mM NaCl/5 mM $MgCl_2$/2 mM $MnCl_2$/50 mM Hepes (pH 7.6)/1 μM nigericin/1 μM valinomycin at a chlorophyll concentration of 20 and 50 $\mu g \cdot cm^{-3}$, respectively, at a light intensity of 40 $W \cdot m^{-2}$ in the presence of various ATP concentrations. The rate of phosphorylation was found to be linear over this 5-min period such that labelling and fluorescence data reflect phosphorylation rate rather than total phosphorylation.

Fluorescence yield of control and phosphorylated thylakoids was measured in the presence of 50 μM DCMU approx. 30 s after the end of the incubation using apparatus described previously [14]. Duplicate samples incubated in the presence of [γ-^{32}P]ATP at a specific activity of 25 $\mu Ci/\mu mol$ were precipitated with 10% trichloroacetic acid. The precipitated proteins were extracted with acetone and subjected to electrophoresis on 10–30% (w/v) polyacrylamide gradient slab gels [7]. Gels were stained with Coomassie Brilliant Blue R, destained, dried and autoradiographed using Kodak X-omat RP X-ray film. ^{32}P incorporation into specific apoproteins was measured by Cerenkov counting of excised gel bands in a Beckman LS 7500 scintillation counter over a 30 min period.

Results

The thylakoid membranes of peas grown under the intermittent light regime are photochemically competent but lack LHCP and are unstacked [16].

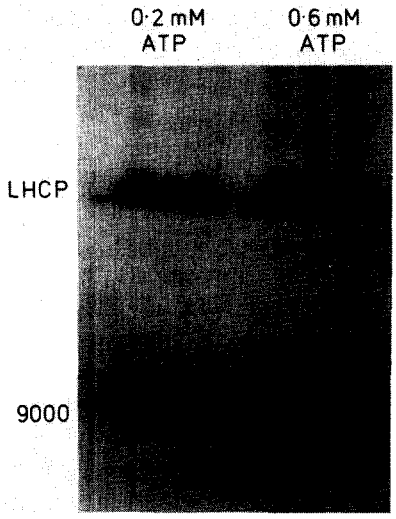

Fig. 1. Autoradiogram of a polyacrylamide gel of intermittent-light thylakoids illuminated in the presence of two different ATP concentrations.

Illumination of intermittent-light plants for 4 h results in synthesis of some LHCP and the appearance of phosphorylation of thylakoid proteins. Fig. 1 shows an autoradiogram of these intermittent-light thylakoids after illumination in the presence of 0.2 and 0.6 mM ATP. Two main phosphoproteins are visible with molecular weights of 9000 and 26000; the former is an unidentified polypeptide, the latter a component of LHCP. A qualitative assessment of the density of the autoradiogram bands suggests that the phosphorylation of the 9 kDa-polypeptide and of LHCP have differing affinities for ATP. In Fig. 2 the measured rates of phosphorylation of the 9 kDa polypeptide and LHCP are plotted as a function of ATP concentration; the concentration of ATP necessary to saturate the rate of phosphorylation of the 9 kDa-polypeptide is clearly greater than that required for LHCP. A qualitatively similar pattern in terms of affinity for ATP is observed in mature thylakoids (Fig. 3).

Fig. 2a and b are presented as examples of the phosphorylation pattern in intermittent-light peas; the ratio of maximal LHCP to 9 kDa-labelling is subject to considerable variation. In the intermittent-light system as this age the chloroplasts are rapidly synthesising LHCP [16], and therefore significant variations in the relative concentrations of

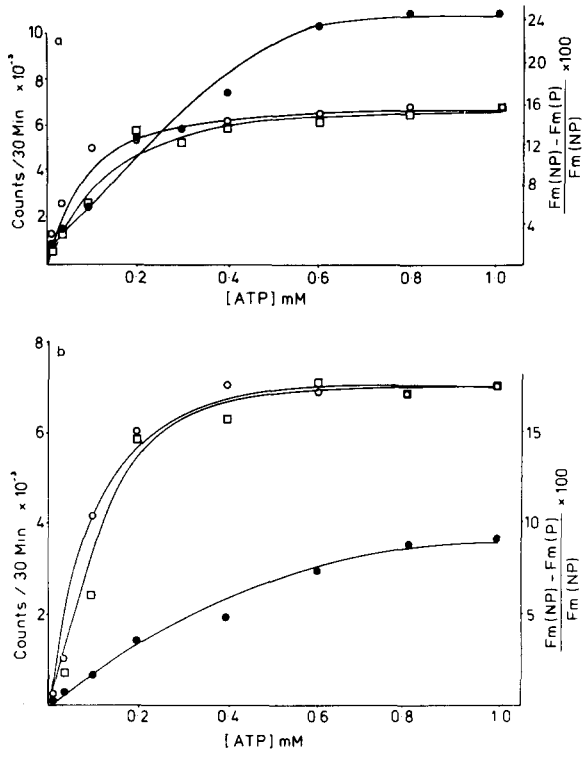

Fig. 2. ^{32}P labelling of LHCP (○) and 9 kDa-polypeptides (●) and decrease in chlorophyll fluorescence yield (□) plotted as a function of ATP concentration in intermittent-light thylakoids. (a) and (b) present two different batches of intermittent-light thylakoids. Labelling is expressed as counts per 30 min of excised gel bands and the fluorescence change as $\{F_m(NP)-F_m(P)\}/F_m(NP)$. P and NP refer to thylakoids illuminated with and without ATP, respectively.

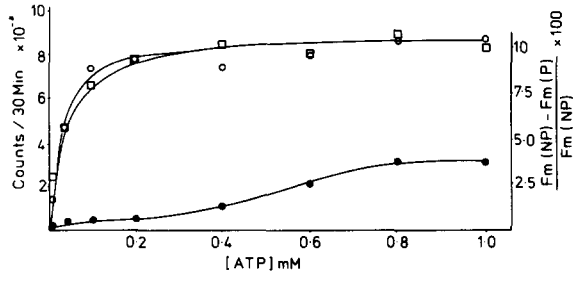

Fig. 3. ^{32}P labelling of LHCP (○) and 9 kDa-polypeptides (●) and decrease in chlorophyll fluorescence yield (□) plotted as a function of ATP concentration in mature thylakoids. Labelling is expressed as counts per 30 min of excised gel bands and the fluorescence change as $\{F_m(NP)-F_m(P)\}/F_m(NP)$. P and NP refer ro thylakoids illuminated with and without ATP, respectively.

TABLE I

RELATIONSHIP BETWEEN THE $S_{0.5}$ VALUES FOR ATP WITH MAXIMUM RATE OF ^{32}P INCORPORATION INTO LHCP AND THE 9 kDa-POLYPEPTIDE.

$S_{0.5}$ values were obtained from data of the type shown in Fig. 2 by measuring the ATP concentration which gives half-maximum rate of phosphorylation. The three values for intermittent-light thylakoids refer to assays made of three different batches of plants.

Thylakoids	$S_{0.5}$ for ATP		Ration of ^{32}P incorporation into LHCP and
	LHCP (mM)	9 kDa (mM)	
Intermittent-light	0.10	0.12	0.17
Intermittent-light	0.12	0.26	0.60
Intermittent-light	0.12	0.32	2.00
Mature	0.04	0.50	2.60

LHCP and the 9 kDa-polypeptide might be expected which would give differences in phosphorylation kinetics. Table I shows the maximum rates of phosphorylation and the ATP concentrations required for half-saturation ($S_{0.5}$). In intermittent-light thylakoids which have a low LHCP/9 kDa ratio, the $S_{0.5}$ for ATP for both polypeptides is approx. 100 μM, whereas in mature thylakoids in which LHCP is the major phosphoprotein the $S_{0.5}$ for ATP of the 9 kDa-polypeptide is approx. 500 μM and that for LHCP approx. 40 μM. The $S_{0.5}$ of the 9 kDa polypeptide is strongly correlated with its concentration relative to LHCP; thus there is a 4-fold increase in $S_{0.5}$ as the LHCP/9 kDa ratio changes from 0.17 to 2.6.

The decrease in yield of chlorophyll fluorescence seen upon illumination with ATP is thought to represent alteration of exciton distribution resulting from LHCP phosphorylation [1,2,12–14]. It was therefore of interest to compare the ATP requirement for phosphorylation of these two polypeptides with the decrease in chlorophyll fluorescence yield. Figs. 2 and 3 show that in each case a close correlation exists between the decrease in fluorescence yield and LHCP phosphorylation, each showing the same dependence on ATP concentration; this correlation was observed regardless of the relative abundance of the LHCP and 9 kDa-phosphoproteins. No fluorescence change could be attributed to the phosphorylation of the 9 kDa-polypeptide. A similar correlation can be seen in Figs. 4 and 5. Fig. 4 is an autoradiogram showing dephosphorylation of intermittent-light thylakoids over a 30 min period. A qualitative assessment of the density of the two main bands indicates a much more rapid dephosphorylation of the LHCP compared with the 9 kDa polypeptide. Fig. 5, which relates the dephosporylation kinetics of LHCP and the 9 kDa-polypeptide with fluorescence yield recovery, confirms this. Again, no correlation between fluorescence yield and degree of phosphorylation of the 9 kDa-polypeptide was found. This confirms the observations of Steinback et al. made on mature thylakoids [3].

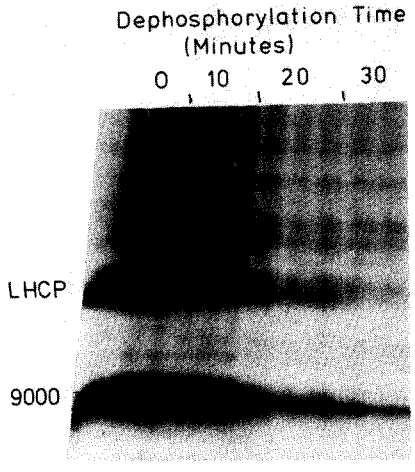

Fig. 4. Autoradiogram of a polyacrylamide gel of intermittent-light thylakoids showing dephosphorylation of LHCP and 9 kDa over a time-course of 30 min. Intermittent-light thylakoids were phosphorylated by illumination for 5 min and allowed to dephosphorylate by incubation in darkness at 20°C.

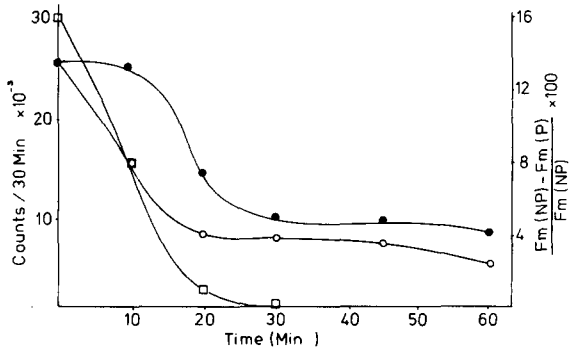

Fig 5. Time-course for dephosphorylation of LHCP (○) and 9 kDa-polypeptide (●), and associated recovery of fluorescence yield (□).

Discussion

The results presented in this paper relate to two important questions concerning phosphorylation of thylakoid proteins: (1) can evidence be found to invoke different protein kinases for the two major phosphoproteins of pea thylakoids; and (2) can the major functional effect of protein phosphorylation (control of excitation distribution) be equated with phosphorylation of the LHCP alone or do the PS II phosphoproteins such as the 9 kDa-species have a role, particularly when the latter is the major phosphoprotein.

In relation to the question of multiple kinases the present data show that the $S_{0.5}$ for ATP is 10-fold higher for phosphorylation of the 9 kDa-polypeptide than for that of LHCP. This could be interpreted as evidence for a different kinase for each of the two phosphoproteins. However, an alternative interpretation is possible. It is clear from Table I that the $S_{0.5}$ for the 9 kDa-polypeptide is not absolute, since in different developmental states values ranging from 500 to 100 μM are found. Significantly, the $S_{0.5}$ is lowest when the 9 kDa/LHCP ratio is highest. Thus, an interpretation of our data is that the two protein substrates are in competition for the same kinase. When the relative concentration of the 9 kDa-polypeptide is high, its affinity for ATP would approach that of LHCP as they compete more equally. In mature pea thylakoids, LHCP is the major phosphoprotein and here the $S_{0.5}$ for the 9 kDa-polypeptide is highest. Protein kinases are two substrate enzymes and the $S_{0.5}$ for one substrate will therefore depend upon the concentration of the other substrate [18]. Thus the differences in the measured $S_{0.5}$ for ATP for phosphorylation of the 9 kDa polypeptide seen in Table I are explainable by expected different concentrations of the protein substrate. Moreover, if the 9 kDa polypeptide and LHCP compete for phosphorylation by the same kinase then the $S_{0.5}$ for ATP for each polypeptide will depend on their relative concentrations. Thus all the data in Table I can be explained in terms of the kinase being a two substrate enzyme which catalyses the phosphorylation of two protein substrates. The data should also be used to highlight the problem of interpreting kinetic experiments on the thylakoid kinase. Thus there is a complete absence of the information (on the concentration of both the protein substrates and the kinase itself and on the interaction between them) that is necessary for analysis of enzyme kinetics.

It has also been clearly shown that the decrease in the maximum yield of chlorophyll fluorescence is associated with phosphorylation of LHCP. Whilst this has been implicated in a number of previous studies [12–4,19], direct proof of the involvement of LHCP (rather than other phosphoproteins) has only previously come from one study of dephosphorylation kinetics [3]. This observation is confirmed here and the same conclusion is derived from examination of the [ATP] requirement for the fluorescence change and the phosphorylation. Moreover, the conclusion that fluorescence yield changes are due only to LHCP phosphorylation applies even in intermittent-light membranes in which the 9 kDa-polypeptide is the major phosphoprotein and in which large ATP-induced fluorescence changes are seen (see Fig. 2a). It should be added that the involvement of phosphorylation of other polypeptides in the fluorescence change cannot be totally excluded although the amount of phosphorylation of these is much less than that of LHCP and the 9 kDa-polypeptide (see, for example, Figs. 1 and 4). The functional significance of the phosphorylation of the 9 kDa-polypeptide (and indeed of the other PS II polypeptides) remains unclear at present. Effects of membrane protein phosphorylation on PS II have been reported which include an increased affinity for DCMU [20], an increase in charge density around Q [21], an increase in stability of B^-, the two electron gate PS II [22] and stimulation of a cyclic electron-transfer pathway of around PS II [23]. It is possible that some or all of these effects are a result of specific PS II polypeptide phosphorylation as opposed to LHCP phosphorylation. Alternatively, their phosphorylation may have a role in membrane assembly, as suggested by Ohad and co-workers [20,24]. In this respect it is interesting to note that in wheat chloroplasts at early stages of development ^{32}P is predominantly incorporated into a 9 kDa-polypeptide [25]. In young tissue, rapid rates of protein synthesis and membrane assembly would be expected, and if phosphorylation has a role in membrane assembly then a correspondingly large degree of PS II phosphory-

lation might be expected.

Finally, the observed affinities for ATP and the apparent competition between polypeptides for phosphorylation may have physiological consequences. In different developmental states or in different species the relative composition of protein complexes in the thylakoid membrane changes [26] and this may well have implications for the operation of regulatory processes based upon reversible protein phosphorylation. Similarly, levels of stromal ATP in vivo may in some circumstances (e.g., during stress) determine the extent of phosphorylation of minor polypeptides such as the 9 kDa-species.

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References

1 Horton, P. (1983) FEBS Lett. 152, 47–52
2 Bennett, J. (1983) Bioch. J. 212, 1–13
3 Steinback, K.E., Bose, S. and Kyle, D.J. (1982) Arch. Biochem. Biophys. 216, 356–361
4 Bennett, J. (1977) Nature 269, 344–346
5 Telfer, A., Allen, J.F., Barber, J. and Bennett, J. (1983) Biochim. Biophys. Acta. 722, 176–181
6 Allen, J.F. and Horton, P. (1981) Biochim. Biophys. Acta 638, 290–295
7 Horton, P., Allen, J.F., Black, M.T. and Bennett, J. (1981) FEBS Lett. 125, 193–196
8 Markwell, J.P., Baker, N.R., Bradbury, M. and Thornber, J.P. (1984) Plant. Phys. 74, 348–354
9 Markwell, J.P., Baker, N.R. and Thornber, J.P. (1983) Photobiochem. Photophys. 5, 201–207
10 Lin, Z.-F., Lucero, H.A. and Racker, E. (1982) J. Biol. Chem. 257, 12153–12156
11 Bennett, J. (1980) Eur. J. Biochem. 104, 85–89
12 Horton, P. and Black, M.T. (1980) FEBS Lett. 119, 141–144
13 Bennett, J., Steinback, K.E. and Arntzen, C.J. (1980) Proc. Natl. Acad. Sci. USA 77, 5253–5257
14 Horton, P. and Black, M.T. (1981) Biochim. Biophys. Acta 653, 53–62
15 Kyle, D.J., Staehelin, L.A. and Arntzen, C.J. (1983) Arch. Biochem. Biophys. 222, 527–541
16 Armond, P.A., Arntzen, C.J., Briantais, J.-M. and Vernotte, C. (1976) Arch. Bioch. Biophys. 175, 54–63
17 Kyle, D.J., Haworth, P. and Arntzen, C.J. (1982) Biochim. Biophys. Acta 680, 336–342
18 Engel, P.C. (1977) Enzyme Kinetics, Chapman and Hall, London
19 Allen, J.F., Bennett, J., Steinback, K.E. and Arntzen, C.J. (1981) Nature 291, 21–25
20 Shocat, S., Owens, G.C., Hubert, P. and Ohad, I. (1982) Biochim. Biophys. Acta. 681, 21–31
21 Kyle, D.J. and Arntzen, C.J. (1983) Photobiochem. Photobiophys. 5, 11–16
22 Jursinic, P.A. and Kyle, D.J. (1983) Biochim. Biophys. Acta 723, 37–44
23 Horton, P. and Lee, P. (1983) FEBS Lett. 162, 81–84
24 Owens, G.C. and Ohad, I. (1983) Biochim. Biophys. Acta 722, 234–241
25 Baker, N.R., Markwell, J.P., Bradbury, M., Baker, M.G. and Thornber, J.P. (1983) Planta 159, 151–158
26 Anderson, J.M. (1982) Molecular and Cellular Biochem. 46, 161–172