PHOTOSYNTHETIC PROTEIN PHOSPHORYLATION IN INTACT CHLOROPLASTS

Inhibition by DCMU and by the onset of CO_2 fixation

John F. ALLEN and John BENNETT
Department of Biological Sciences, University of Warwick, Coventry, CV4 7AL, England

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1. Introduction

Chloroplasts contain a membrane-bound protein kinase which brings about phosphorylation of a number of thylakoid proteins [1], including the chlorophyll *a/b* binding protein of the light-harvesting chlorophyll–protein complex, LHCP [2]. Although ATP is the substrate for this protein phosphorylation, intact chloroplasts will incorporate [^{32}P]orthophosphate into thylakoid proteins by virtue of their possession of an endogenous pool of adenine nucleotides and their capacity for photosynthetic phosphorylation of ADP. Protein phosphorylation in intact chloroplasts is light-dependent not only because of its dependence on photophosphorylation but also because the protein kinase is itself activated by illumination of the thylakoids to which it is bound [3].

Published studies of chloroplast protein phosphorylation have hitherto been carried out on thylakoids in the absence of added electron acceptors or on intact chloroplasts incapable of CO_2 fixation. The investigation we describe in this letter was designed to establish which type of photosynthetic electron transport (cyclic or non-cyclic) activates protein phosphorylation in intact chloroplasts, and to see whether protein phosphorylation accompanies CO_2 fixation in intact chloroplasts when they are supplied with CO_2 to provide a physiological electron acceptor for non-cyclic electron transport.

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid; LHCP, light-harvesting chlorophyll *a/b* binding protein; RM, resuspension medium; SDS, sodium dodecylsulphate; tricine, *N*-tris-(hydroxymethyl)methylglycine

2. Experimental

Intact chloroplasts were isolated from shoots of 9–11 day-old peas (*Pisum sativum* var. Feltham First) by a method based on that in [4], using a Polytron homogenizer and a single centrifugation at 4000 × *g* for 1 min. The pea shoots (30 g) were given two treatments in the homogenizer, each lasting 4 s, in 200 ml of a medium containing sucrose (0.35 M), EDTA (2 mM), ascorbate (2 mM) and Hepes (25 mM) at pH 7.6. The pellet was resuspended in a small volume of a medium (sorbitol RM) containing sorbitol (0.33 M), EDTA (2 mM), ascorbate (2 mM), $MgCl_2$ (1 mM), $MnCl_2$ (1 mM) and Hepes (50 mM) at pH 7.6. For table 1, a pellet was also resuspended in a medium ('KCl–RM') containing KCl (0.2 M), $MgCl_2$ (6.6 mM) and tricine (66 mM) at pH 8.3. The proportion of intact chloroplasts in each preparation was estimated as relative impermeability to ferricyanide [5].

Reactions were carried out in the vessel of an oxygen electrode (Rank Bros., Bottisham) which was illuminated by a slide projector (150 W) carrying an orange (Cinemoid 5A) filter. The reaction medium was 'sorbitol RM' (or 'KCl–RM' where indicated in table 1) to which the following had been added to the stated final concentrations: $NA_4P_2O_7$ (5 mM), ATP (1 mM), $NaHCO_3$ (10 mM) and [^{32}P]orthophosphate (150 μCi) for CO_2 fixation; $K_3Fe(CN)_6$ (2 mM) for electron transport measurements. NH_4Cl (5 mM), catalase (5000 units) and D,L-glyceraldehyde (10 mM) were also present where indicated. The inclusion of $Na_4P_2O_7$ and ATP was found to be necessary in order to obtain appreciable rates of CO_2-dependent O_2 evolution [6,7]. The total volume of each reaction mixture was 2.0 ml (5.0 ml for fig.3) and chlorophyll (chl) was 50 μg/ml.

In the experiment of fig.1,2 each sample (0.5 ml) of suspension was withdrawn after 10 min illumination. In the experiment of fig.3, 100 μl aliquots of suspension were withdrawn at intervals from the reaction vessel, and the plunger of the oxygen electrode was adjusted to eliminate the air which displaced each aliquot. Each sample was immediately mixed with 4.5 ml cold trichloroacetic acid (10%) and left on ice for ⩾1 h. Each mixture was then centrifuged at 4000 × *g* for 10 min, the pellet resuspended in 80% acetone (2 ml) and left in the cold overnight. The suspension was centrifuged as before, and the pellet dried under a stream of N_2 gas. The protein pellets were resuspended and subjected to SDS–polyacrylamide gel electrophoresis as in [2]. The amount of labelling of the LHCP protein was measured by scintillation counting of the cut-out LHCP band.

ATP and bovine catalase were from the Sigma Chemical Co., [^{32}P]orthophosphate was from the Radiochemical Centre, Amersham.

3. Results

Intact chloroplasts that had been used in studies of protein phosphorylation [1] were isolated in media appropriate for studies of chloroplast protein synthesis. Table 1 shows that such a medium (KCl–RM) is inappropriate for photosynthetic measurements. Only 48% of chloroplasts suspended in 'KCl–RM' were intact, and they showed no CO_2-dependent O_2 evolution. When osmotically shocked, their lack of response to the uncoupler NH_4Cl suggests that they are already largely uncoupled. In contrast, chloroplasts suspended in a conventional sorbitol medium (sorbitol RM) gave a higher intactness value (73%), showed CO_2-dependent O_2 evolution when intact and stimulation of electron transport by NH_4Cl when broken. All other results presented here were obtained with photosynthetically competent chloroplasts suspended in the sorbitol medium.

Table 1
Effect of suspension medium on ferricyanide-dependent and CO_2-dependent O_2 evolution (μmol . (mg chl)$^{-1}$. h^{-1}) in pea chloroplasts

Electron acceptor	Sorbitol RM	KCl–RM
Ferricyanide		
Chloroplasts unshocked		
Without NH_4Cl	7.9	41.0
With NH_4Cl	32.3	41.0
Chloroplasts shocked		
Without NH_4Cl	18.5	72.3
With NH_4Cl	118	79.4
CO_2 (as $NaHCO_3$)	35.5	−6.6
Apparent proportion of intact chloroplasts	73%	48%

'Sorbitol RM' and 'KCl–RM' are defined in the text

The autoradiograph reproduced in fig.1 shows the effect of different DCMU concentrations on the intensity of labelling resulting from phosphorylation of different chloroplast proteins. Since [^{32}P]orthophosphate will label proteins in intact chloroplasts only [1], the bands visible in fig.1 must be of proteins phosphorylated in the intact chloroplasts of the preparation. The densest labelling occurred in the 26 000 M_r band of the LHCP protein [2] which was among the bands in which labelling was most susceptible to inhibition by DCMU. At 10 μM DCMU, and in the absence of catalase, labelling of the LHCP protein was almost completely absent, while under the same conditions labelling of higher M_r bands was still apparent. Fig.1 also shows that labelling of chloroplast proteins by [^{32}P]orthophosphate is stimulated by the presence of catalase, while catalase has no influence on the sensitivity of labelling to DCMU.

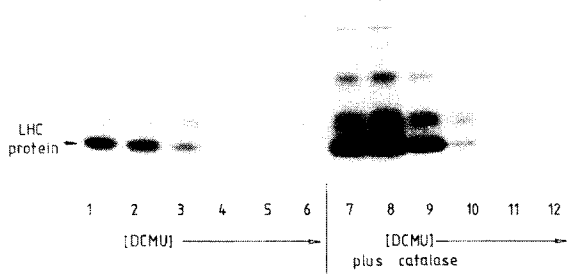

Fig.1. Effects of DCMU and catalase on protein phosphorylation in intact chloroplasts. Autoradiograph of polyacrylamide gel in which pea chloroplast proteins had been separated by electrophoresis in the presence of SDS. Tracks 1–6 record labelling in intact chloroplasts incubated in the light in the presence of [^{32}P]orthophosphate with successively higher concentrations of DCMU and in the absence of catalase. Tracks 7–12 also record effects of increasing DCMU concentration, but in the presence of added catalase (5000 units). The DCMU concentrations were as follows: (1,7) zero (controls); (2,8) 0.1 μM; (3,9) 0.3 μM; (4,10) 1 μM; (5,11) 3 μM; (6,12) 10 μM.

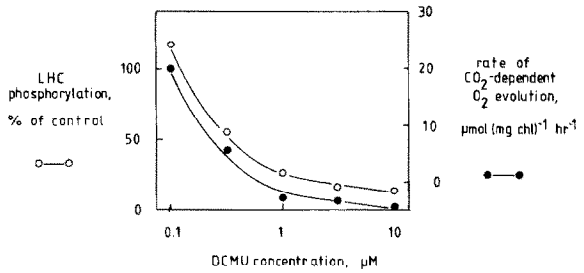

Fig.2. Effect of DCMU on CO_2-dependent O_2 evolution and LHCP phosphorylation in intact pea chloroplasts. The phosphorylation data were obtained as in fig.1 (in the presence of catalase). The control level of phosphorylation was 640 cpm/LHCP band. CO_2-dependent O_2 evolution was measured in the presence of $NaHCO_3$, ATP, pyrophosphate and catalase as in section 2.

The effect of catalase on protein phosphorylation may result from its suppression of an inhibitory effect of H_2O_2. H_2O_2 may be generated in a Mehler reaction in the broken chloroplasts of the preparation [8].

Fig.2 shows the effects of DCMU on LHCP protein phosphorylation and on CO_2-dependent O_2 evolution in the same chloroplast preparation. The level of protein phosphorylation in the presence of 0.1 μM DCMU was greater than that obtained in the absence of DCMU, while higher DCMU concentrations caused inhibition of phosphorylation. Inhibition giving 50% of the highest level of phosphorylation was obtained at 0.2 μM DCMU, a value which indicates that electron transport from water through photosystem II is required for activation of phosphorylation [9]. In the same experiment (fig.2) CO_2-dependent O_2 evolution was also found to be inhibited by 50% by 0.2 μM DCMU, in agreement with the results in [10].

Fig.3(a) shows the onset of CO_2-dependent O_2 evolution and the changing levels of LHCP protein phosphorylation which accompanied it. The maximum rate of CO_2-dependent O_2 evolution occurred only after a 2–3 min lag-phase. In contrast, the maximum rate of LHCP phosphorylation was seen in the first 2 min illumination, before CO_2-dependent O_2 evolution had begun at an appreciable rate. The onset of CO_2-dependent O_2 evolution was accompanied by a decline in the rate of protein phosphorylation, while subsequent photosynthetic activity (after ⩾5 min illumination) was accompanied by a net dephosphorylation of LHC protein. This result indicates that protein phosphorylation is inhibited during CO_2-fixation. If this is so then a specific inhibitor of the Calvin

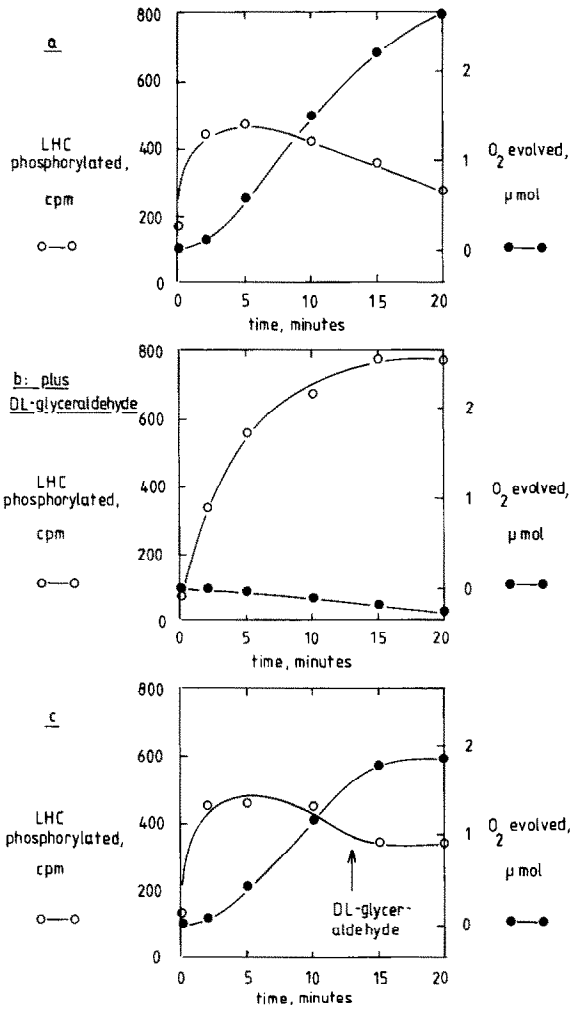

Fig.3. The time-course of CO_2-dependent O_2 evolution (•) and of phosphorylation of LHC protein (o) in intact pea chloroplasts (a) in the absence and (b) in the presence of D,L-glyceraldehyde (10 mM), an inhibitor of CO_2-fixation. In (c) D,L-glyceraldehyde was added after 13 min illumination. O_2 evolution proceeded at a maximum rate of 36 μmol . (mg chl)$^{-1}$. h^{-1}.

cycle should increase the rate of protein phosphorylation and avert net dephosphorylation. Fig.3(b) shows the corresponding time-courses obtained in the presence of such an inhibitor, D,L-glyceraldehyde [12]. As predicted, the level of protein phosphorylation was elevated by D,L-glyceraldehyde, and the level continued to rise throughout the experiment. CO_2-dependent O_2 evolution was completely absent. Fig.3(c) shows that addition of D,L-glyceraldehyde even after 13 min CO_2-fixation arrests the subsequent decline in the level of LHCP phosphorylation.

4. Discussion

We conclude in [13] that the thylakoid protein kinase is activated by reduced plastoquinone and inactivated by oxidized plastoquinone. Part of the evidence for this conclusion is the ability of electron acceptors such as methyl viologen and ferricyanide to inhibit the kinase in an assay involving isolated thylakoids. The presence of an artificial electron acceptor for photosystem I tends to oxidize plastoquinone and thus inhibit the kinase. The results in fig.3 indicate that the physiological electron acceptor, CO_2, has a similar inhibitory effect in intact chloroplasts.

A second possibility is that the inhibition of kinase activity seen in fig.3(a) is also due to competition between the kinase and the Calvin cycle for ATP. However, this possibility is excluded by the fact that the K_m for ATP of the kinase is 90 μM [14], while the K_m for ATP of phosphoribulokinase (EC 2.7.1.19) is 280 μM [15]. Thus a shortage of ATP is likely to inhibit the Calvin cycle before it inhibits the protein kinase.

The inverse relationship between protein kinase activity and CO_2-fixation may be important physiologically. It has been reported that phosphorylation of the LHCP is responsible for the redistribution of absorbed excitation energy to photosystem I at the expense of photosystem II [14]. Thus the proportion of absorbed excitation energy transferred to photosystem I will be greater during the lag phase (when the level of LHCP phosphorylation is high) than during rapid CO_2 fixation. This mechanism could allow the rate of cyclic photophosphorylation to be enhanced during the lag phase, at a time when the rate of non-cyclic photophosphorylation is limited by the rate of NADPH re-oxidation. In [16] the lag phase appeared shortened when the ATP/ADP ratio was increased. Thus transient phosphorylation of the LHCP may be important for the rapid return of dark-adapted chloroplasts to maximal rates of CO_2 fixation.

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