CHLOROPLAST PROTEIN PHOSPHORYLATION AND CHLOROPHYLL FLUORESCENCE QUENCHING

ACTIVATION BY TETRAMETHYL-p-HYDROQUINONE, AN ELECTRON DONOR TO PLASTOQUINONE

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When tetramethyl-p-benzoquinone (TMQ) is reduced to tetramethyl-p-hydroquinone (TMQH₂) by NaBH₄, TMQH₂ will act as an electron donor in isolated chloroplasts. The resulting electron transport is highly sensitive to inhibition by 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), and the site of donation is inferred to be plastoquinone, in agreement with previous findings. In contrast, when TMQ is added to chloroplasts with ascorbate as reductant, the resulting electron transport is relatively insensitive to DBMIB, and so plastoquinone is assumed not to be involved. In darkness, TMQH₂ activates the chloroplast protein kinase that phosphorylates the light-harvesting chlorophyll a/b-protein (LHCP), while TMQ with ascorbate does not. TMQH₂ also activates ATP-dependent chlorophyll fluorescence quenching to a much greater extent than does TMQ with ascorbate. These findings are explained by the recent proposal that phosphorylation of LHCP is activated by reduced plastoquinone. They are therefore evidence for plastoquinone-regulated protein phosphorylation as a mechanism for self-adjustment of distribution of excitation between the two light reactions of photosynthesis.

Introduction

Isolated chloroplasts possess a protein kinase which catalyzes the light-dependent and DCMU-sensitive phosphorylation of a number of thylakoid proteins [1–3]. ATP-dependent quenching of chlorophyll fluorescence in isolated chloroplasts is now thought to be a consequence of phosphorylation of one of these proteins, LHCP [4,5].

Studies of activation by single-turnover flash illumination and of inhibition by site-specific inhibition of electron transport [5] support the hypothesis [5, 6] that reduced plastoquinone activates LHCP phosphorylation, thereby redistributing excitation energy to PS I. Oxidized plastoquinone is thought to inhibit kinase activity and to lead to net dephosphorylation of LHCP with consequently decreased transfer of excitation energy to PS I. Potentiometric redox titration of ATP-dependent chlorophyll fluorescence quenching [7] and of LHCP phosphorylation [8] confirms that these processes are regulated by the redox state of a two-equivalent carrier with the mid-point potential of plastoquinone (Em,7.8 ≈ 0 mV). The proposed involvement of plastoquinone in regulation of these reactions would enable protein phosphorylation to mediate a negative-feedback control of distribution of excitation energy between photosystems, thus explaining the adaptations to...
changing light quality that are manifested as State 1-State 2 transitions.

In this paper we report studies on the use of an artificial electron donor, TMQH₂. TMQH₂ has been reported to be a specific donor to plastoquinone [9, 10]. If this is the case, and if LHCP phosphorylation is controlled by the plastoquinone redox state, then TMQH₂ might be expected to activate LHCP phosphorylation and associated chlorophyll fluorescence changes even in darkness or in the presence of DCMU.

Materials and Methods

Intact pea chloroplasts were isolated in a sorbitol medium, as previously described [11]. Electron transport in broken chloroplasts was measured as oxygen exchange in an illuminated oxygen electrode [11], using a hypotonic medium containing sorbitol (0.1 M), MgCl₂ (5 mM), NaCl (5 mM), Hepes (50 mM) at pH 7.6, and chloroplasts equivalent to 100 µg of chlorophyll in a final volume of 2.0 ml. Other constituents are indicated in the figure legend for the experiment concerned.

LHCP phosphorylation was carried out in 1.0 ml of the same medium and with the same chlorophyll concentration, though ATP (0.1 mM) and high specific activity [γ-³²P]ATP (10 µCi) were also present. The reaction was stopped after 4 min by addition of 50 µl of trichloroacetic acid (100%, w/v). Samples were prepared and used for SDS-polyacrylamide gel electrophoresis as described in Ref. 11. Chlorophyll fluorescence (Fₘₐₓ) was measured as detailed in Ref. 8.

TMQ (50 mM in ethanol) was reduced to TMQH₂ with NaBH₄ (about 2 mg added to 0.5 ml of solution). After standing the mixture on ice for 5 min, excess NaBH₄ was removed by addition of concentrated HCl (5 µl) [9].

[γ-³²P]ATP was obtained from the Radiochemical Centre, Amersham; DCMU, DCIP and TMQ were from Sigma, Poole.

Results and Discussion

Since its introduction [12], DBMIB has been used extensively as an inhibitor of photosynthetic electron transport. At sub-micromolar concentrations it inhibits oxidation of plastoquinone specifically. Fig. 1a illustrates this action of DBMIB in pea chloroplasts; half-maximum inhibition of whole-chain electron transport (water to methyl viologen) occurred at less than 50 nM DBMIB, while the corresponding PS I-mediated electron transport from ascorbate with DCIP was inhibited by less than 20% even at 10 µM DBMIB. In agreement with previous reports [9,10], Fig. 1b shows that PS I-mediated electron transport from TMQH₂ retains the high DBMIB sensitivity (Kᵢ < 50 nM) of whole-chain electron transport, and therefore includes plastoquinone oxidation as a component step. Contrary to the results of Izawa and Pan [9], we obtained appreciable electron-transport rates with TMQ and ascorbate as a donor couple. This partial reaction was insensitive to DBMIB (Fig. 1b) and therefore does not involve plastoquinone. Fig. 1c shows that electron transport from TMQH₂ is sensitive to inhibition by DBMIB but insensitive to inhibition by DCMU. Since plastoquinone is the major component between these two inhibition sites, it is concluded that TMQH₂ is a specific donor to plastoquinone.

We have confirmed the observation [9] that oxygen uptake in the system TMQH₂ → plastoquinone → PS I → methyl viologen → O₂ is slowed by addition of superoxide dismutase or catalase, and have found that this is also true in the system ascorbate/TMQ → PSI → methyl viologen → O₂ (results not shown). We conclude that TMQH₂ (like ascorbate) reduces O₂⁻ to H₂O₂ and that two O₂ molecules are therefore consumed per electron pair transferred from TMQH₂ to O₂ via PS I [14].

Fig. 2 is an autoradiograph of pea chloroplast phosphoproteins separated by polyacrylamide gel electrophoresis. Labelling of the molecular weight 26 000 LHCP band was clearly light dependent (tracks 1 and 2) and DCMU sensitive (tracks 1, 3 and 4). The effect of TMQH₂ was to activate phosphorylation in the dark (track 6), thus indicating that dark reduction of plastoquinone can activate the protein kinase. In the light, TMQH₂ decreased the level of LHCP phosphorylation (track 5) compared with that of the control (track 1). We tentatively attribute this effect to oxidation of TMQH₂ by PS I during the course of the incubation. This would also account for the failure of TMQH₂ to restore phosphorylation to DCMU-inhibited chloroplasts in the light (track 7). Since the standard redox potential of TMQ/TMQH₂
Fig. 1. (a and b) Electron transport, measured as oxygen uptake, as function of DBMIB concentration. (a) (o-o) Ascorbate → DCIP → methyl viologen → O₂; (•-•) H₂O → methyl viologen → O₂; (b) (o-o) Ascorbate → TMQ → methyl viologen → O₂; (•-•) TMQH₂ → methyl viologen → O₂. Reaction conditions as described in the text, with the following additions. All curves; methyl viologen (50 μM), NaN₃ (5 mM). (a) Upper curve: DCMU (5 μM), DCIP (0.1 mM), ascorbate (2 mM). (b) Upper curve: DCMU (5 μM), TMQ (0.5 mM), ascorbate (2 mM). (b) Lower curve: DCMU (5 μM), TMQH₂ (0.5 mM). Control rates of O₂ uptake, in μmol/mg Chl per h, were as follows. (a) Lower curve, 65; upper curve, 109. (b) Lower curve, 189; (b) upper curve, 61. (c) Oxygen electrode trace obtained under the conditions for a (lower curve) and in the absence of DBMIB. Subsequent additions gave the final concentrations quoted above for a and b. DBMIB was added where indicated to give a final concentration of 20 μM. Numbers are rates of O₂ uptake, in μmol/mg Chl per h, for adjacent parts of the trace. The final drop in rate resulted from exhaustion of the O₂ in the medium.

[9] is close to that of plastoquinone [7] at about 0 mV, it is to be expected that protein phosphorylation is sensitive even to small changes in the redox state of TMQ/TMQH₂. Track 8 of Fig. 2 shows that DCMU does not inhibit activation by TMQH₂ in the dark.

We conclude from Fig. 2 that TMQH₂ activates protein phosphorylation in darkness by reducing plastoquinone, and that it does not activate phosphorylation in the presence of DCMU in the light, since it is then unable to maintain plastoquinone in the fully reduced state. In contrast, the non-specific reductant dithionite has been shown to activate phosphorylation both in darkness and in the presence of DCMU in the light [5]. The specificity of TMQH₂ (Fig. 1) strengthens the conclusion that it is the redox state of plastoquinone itself that regulates chloroplast protein phosphorylation.

Table I indicates that conditions for LHCP phosphorylation are also conditions for chlorophyll fluorescence quenching. Thus, both processes are activated by TMQH₂ in the dark, and inhibited by it in the light. TMQ with ascorbate inhibits in the light and does not activate completely in the dark, which we attribute to a by-passing of plastoquinone by this donor couple (donation is DBMIB insensitive, Fig. 1) and to oxidation of plastoquinone by TMQ. Ascorbate (Eₘ,₇ = +58 mV) [15] is expected only partially to reduce TMQ (Eₘ,₇ = +5 mV) [10] and therefore to provide only a slow electron flow through plastoquinone. Most donation by ascorbate via TMQ is presumably at a secondary site, closer to PS I. For the same reason DCIP/DCIPH₂ (Eₘ,₇ = 217 mV) [15] is not expected to cause activation, and should inactivate in the light. This is the case for protein phosphorylation (Table I); ATP-dependent chloro-
phyll fluorescence quenching was not measured in the presence of DCIP because of the strong ATP-independent quenching then obtained.

In Table I there is some discrepancy in the absolute values, particularly in the effects of TMQ with ascorbate. It is likely that these reflect no more than necessary differences in conditions for the two sets of measurements. Table I would then be consistent with the inseparability of LHCP phosphorylation and ATP-dependent chlorophyll fluorescence quenching in chloroplast membranes [4,5], and, by virtue of the specificity of TMQH₂ as an electron donor (Fig. 1), it reinforces our conclusion [5–8] that the plastoquinone redox state is an essential regulator of these processes.

Scheme I presents our interpretation of the results.
TABLE I
LHCP PHOSPHORYLATION AND ATP-DEPENDENT CHLOROPHYLL FLUORESCENCE QUENCHING, EACH EXPRESSED AS A PERCENTAGE OF THE CONTROL VALUE

Reaction conditions and final concentrations for LHCP phosphorylation were as described for fig. 2. The control value is 230 cpm in the cut-out LHCP band. For fluorescence measurements, chloroplasts were incubated as described in Ref. 5 for 10 min. Fm was measured in the presence of DCMU and dithionite which were added at the end of the incubation. The control fluorescence decrease was 21%. L, light; D, dark.

<table>
<thead>
<tr>
<th>Condition</th>
<th>LHCP phosphorylation (% of control)</th>
<th>ATP-dependent quenching of chlorophyll fluorescence (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L (control)</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>D</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>L, DCMU</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>D, DCMU</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>L, TMQH₂</td>
<td>46</td>
<td>71</td>
</tr>
<tr>
<td>D, TMQH₂</td>
<td>78</td>
<td>100</td>
</tr>
<tr>
<td>L, DCMU, TMQH₂</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>D, DCMU, TMQH₂</td>
<td>58</td>
<td>98</td>
</tr>
<tr>
<td>L, TMQ, ascorbate</td>
<td>7</td>
<td>32</td>
</tr>
<tr>
<td>D, TMQ, ascorbate</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>L, DCMU, TMQ, ascorbate</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>D, DCMU, TMQ, ascorbate</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>L, DCIP, ascorbate</td>
<td>5</td>
<td>--</td>
</tr>
<tr>
<td>D, DCIP, ascorbate</td>
<td>5</td>
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</tbody>
</table>

DCIP + ascorbate

\[ \text{DMCU} \downarrow \Downarrow \text{DBMIB} \]

\[ \text{H₂O} \rightarrow \text{PS II} \rightarrow \text{plastoquinone pool} \rightarrow \text{PS I} \rightarrow \text{methyl viologen} \rightarrow O₂ \]

\[ \text{protein kinase} \]

\[ \text{LHCP phosphorylation} \downarrow \]

\[ \text{increased transfer of excitation to PS I} \downarrow \]

\[ \text{decreased Chl} \text{II fluorescence} \]

Scheme I. A scheme depicting effects of electron donors (TMQH₂, TMQ or DCIP plus ascorbate) and inhibitors (DCMU and DBMIB) on activation of LHCP phosphorylation and on associated quenching of chlorophyll fluorescence. The scheme is explained further in the text. Chl, chlorophyll.

Acknowledgements

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and on associated quenching of chlorophyll fluorescence. The conclusion is consistent with the following hypothesis, which we describe in greater detail elsewhere [5–8].

Physiological control of the plastoquinone redox state is exerted by the two photosystems in such a way that any imbalance in their activities will cause net oxidation or reduction of plastoquinone. A consequence of this imbalance is phosphorylation-mediated redistribution of excitation energy which will tend to eliminate the imbalance that produced it. Such a control mechanism would increase the photochemically useful fraction of total absorbed excitation energy to a value both high and constant over an otherwise unfavourable range of wavelengths.
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