Chapter 6
THE ROLE OF SECONDARY QUINONE ACCEPTOR $Q_B$ OF PHOTOSYSTEM II IN PHOTOCINHIBITION OF ISOLATED WHEAT CHLOROPLASTS

Content

6.1 Introduction

6.2 Materials and Methods
   6.2.1 Photoinhibition of chloroplasts
   6.2.2 Recording of chlorophyll fluorescence transients
   6.2.3 Measurement of electron transport activity
   6.2.4 Determination of photo-oxidation of pigments

6.3 Results and Discussion
   6.3.1 In vivo chlorophyll fluorescence
   6.3.2 Electron transport studies
   6.3.3 Photo-oxidation of pigments
6.1 Introduction

Exposure of green leaves or isolated chloroplasts to high light intensities damages the photosynthetic apparatus specifically by impairing the photosystem II activity (Barenyi and Krause 1985, Cleland and Critchley 1985, Critchley 1981, Critchley and Smillie 1981, Krause et al. 1985, Nedbal et al. 1986, Powles and Bjorkman 1982). Studies of the inhibition of $Q_B$-dependent and $Q_B$-independent activities of higher plant thylakoids (Cleland and Critchley 1985, Ohad et al. 1985) have been used to identify the primary site of the damage. The results indicate that the $Q_B$-dependent activity is lost prior to the $Q_B$-independent reaction center activity which suggests that the initial site of photoinhibition damage is probably at the $Q_B$ position. The damage at $Q_B$ position is, however, followed rapidly by damage to the photosystem II reaction center (Kyle 1987). There is no agreement over the primary site of damage due to photoinhibition and several other sites between $Z$ and $Q_A$ have been proposed (Allakhverdiev et al. 1987, Callahan et al. 1986, Demeter et al. 1987, Styring et al. 1990). One of the reasons for this decrease in $Q_B$-dependent activity could be the light induced selective degradation of the Dl protein as suggested by Ohad et al. (1986). Damage to the Dl protein depends on the time...
dependent concentration of semiquinone anions during photoinhibition of chloroplasts. Kyle (1987) has proposed a model for photoinhibition damage to the D1 protein which involves the semiquinone anions along with superoxide and hydroxyl free radicals. As DCMU is known to bind to the D1 protein, we have used DCMU during photoinhibition of isolated wheat chloroplasts. DCMU binding to D1 protein should keep the site blocked which might reduce the formation of semiquinone anions which are potentially damaging to the PSII complex of chloroplasts. We have also used DBMIB in absence of DCMU during photoinhibition to reduce the plastoquinone pool which would result in accumulation of semiquinone anion radicals.

6.2 Materials and Methods

Wheat seedlings were grown on vermiculite supplemented with half strength Hoagland's solution. Chloroplasts were isolated from 8-10 days old seedlings as described earlier (see Chapter 2).

6.2.1 Photoinhibition of chloroplasts

Photoinhibitory treatment to the isolated chloroplasts (100 pg Chl/ml) were given in a jacketed glass container as described in Chapter 3. All the treatments to chloroplasts
were given at 2250 W/m² at 25°C for different periods of time up to 40 min. DCMU or DBMIB was added to give 1 µM concentration before photoinhibition treatment wherever indicated.

6.2.2 Recording of chlorophyll fluorescence transients

Fluorescence induction curves of chloroplasts were recorded at room temperature for 6 sec. on a transient recorder (TR1, Hansatech, England) as described earlier (see Chapter 4). All the fluorescence induction measurements were done in presence of 20 µM DCMU. 20 mM hydroxylamine (NH₂OH) was added to the sample as an electron donor wherever indicated.

6.2.3 Measurement of electron transport activity

Measurement of rates of photosynthetic electron transport was performed at 25°C in presence of silicomolybdate as acceptor on an oxygen electrode (CBlD, Hansatech, England). The reaction mixture contained 100 mM sucrose, 40 mM Hepes-KOH (pH 7.0) 15 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂ and chloroplast equivalent to 50 µg Chl/ml. DCMU, silicomolybdate and potassium ferricyanide were also added to the reaction mixture just before the assay to give final concentrations of 5 µM, 0.1 mM and 0.5 mM respectively.
6.2.4 Determination of photo-oxidation of pigments

The content of Chl a, Chl b and carotenoids in chloroplasts after exposure to high light stress for different periods of time was determined according to Arnon (1949).

6.3 Results and Discussion

6.3.1 In vivo chlorophyll fluorescence

Increase in the duration of photoinhibition of chloroplasts resulted in a gradual reduction in \( F_v/F_m \) ratio (Fig. 6.1). \( F_v/F_m \) ratio is a quantitative measure of the photochemical efficiency of photosystem II complex (Kitajima and Butler 1975) and photon yield of oxygen evolution at high light intensities (Bjorkman and Demmig 1987, Demmig and Bjorkman 1987, Adams III et al. 1990). The decrease in \( F_v/F_m \) was more when the thylakoids were exposed to high light intensity in presence of DBMIB and it was less when DCMU was present during photoinhibition. This decrease coincided well with the reduction in variable fluorescence (Fig. 6.2). The reduction in variable fluorescence was brought about by decrease in maximal chlorophyll fluorescence as there was no significant change in \( F_0 \). Reduction in \( F_v \) on exposure of chloroplasts to high light intensity reflects a decrease in photoreduction of the primary quinone acceptor \( Q_A \) (Bjorkman 1987a). Photoreduction of \( Q_A \) also depends on reduction
Fig. 6.1 Effect of presence of DCMU and DBMIB on the $F_v/F_m$ ratio of wheat chloroplasts during photoinhibition. Chloroplasts (100 ug Chl/ml) were illuminated at 2250 W/m². O: no addition, ●: 1 µM DCMU, △: 1 µM DBMIB.
Fig. 6.2 Effect of DCMU and DBMIB on variable chlorophyll fluorescence ($F_v$) on photoinhibition of isolated wheat chloroplasts. ○: chloroplasts photoinhibited without any addition, ●: photoinhibited in presence of 1 µM DCMU, △: photoinhibited in presence of 1 µM DBMIB.
of \( P680^+ \) after the electron is lost from the reaction center chlorophyll. Stress-induced alterations on the donor side of PSII might result in decrease in efficiency of water oxidation. In such conditions, electrons may not be available for reduction of \( P680^+ \) which is likely to result in reduction of variable chlorophyll fluorescence. Hydroxylamine has been shown to donate electrons to the reaction center bypassing the oxygen evolving complex. A recovery of variable chlorophyll fluorescence in photoinhibited chloroplasts was observed in presence of hydroxylamine irrespective of the presence or absence of DCMU or DBMIB (Table 6.1). Restoration of variable fluorescence in presence of hydroxylamine might be due to a damage on the donor side of PSII complex. Thus it seems likely that photoinhibition damage of photosystem II is not only localized either to the acceptor or the donor side of PSII.

Reduction in \( \frac{F_v}{F_m} \) on exposure of chloroplasts to excessive light may be caused by two concomitant events: (i) a decrease in the rate of photochemistry of PSII, and (ii) an increase in the rate constant for non-radiative (thermal) dissipation or preferential transfer of excitation energy to PSI centers. The rate constants for PSII photochemistry (\( K_p \)), non-radiative energy dissipation and energy transfer
Table 6.1

Hydroxylamine induced recovery of variable chlorophyll fluorescence of wheat chloroplasts photoinhibited at 2250 W/m² in presence and absence of DCMU or DBMIB. Fluorescence transients were recorded in presence of 20 mM hydroxylamine (NH₂OH) as a donor to photosystem II of chloroplasts.

<table>
<thead>
<tr>
<th>Period of light treatment (min)</th>
<th>Variable chlorophyll fluorescence (rel. units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No addition</td>
</tr>
<tr>
<td></td>
<td>with NH₂OH</td>
</tr>
<tr>
<td>0</td>
<td>84±1</td>
</tr>
<tr>
<td>10</td>
<td>69±3</td>
</tr>
<tr>
<td>20</td>
<td>61±2</td>
</tr>
<tr>
<td>30</td>
<td>51±2</td>
</tr>
<tr>
<td>40</td>
<td>49±2</td>
</tr>
</tbody>
</table>
Fig. 6.3 Effect of high photoinhibition of isolated wheat chloroplasts on the rate constant for primary photochemistry of PSII ($K_P$). ○: no addition, ●: 1 μM DCMU, Δ: 1 μM DBMIB.
to PSI ($K_{DT}$) were affected on photoinhibition of chloroplasts in our experiments irrespective of the presence or absence of DCMU or DBMIB. Fig. 6.3 shows a reduction in $K_p$ which indicates a decrease in the efficiency of primary photochemistry of PSII complex (Ogren and Oquist 1984, Demmig and Bjorkman 1987). The increase in $K_{DT}$ (Fig. 6.4) would indicate either an increased rate of de-excitation by means of non-radiative decay mechanisms or increased rate of transfer of excitons preferentially to the PSI complex at high light intensities (Bjorkman 1987a, 1987b).

Increase in $K_{DT}$ which indicates transfer of excitation energy to PSI complex could also cause a quenching of $F_m$. Such mechanisms are also considered to be ways of protection of the PSII centers from over-excitation because they result in diversion of excess excitation energy to the PSI centers. It is apparent from Fig. 6.3 and 6.4 that the reduction in $K_p$ and increase in $K_{DT}$ was less when chloroplasts were photoinhibited in presence of DCMU. On the other hand, presence of DBMIB during photoinhibition results in enhancement of the damage. The results thus suggest that DCMU extends a protection of chloroplasts from high light stress. Our results are also in agreement with that of Kyle et al. (1984) who have demonstrated that when Chlamydomonas
Fig. 6.4 Effect of high photoinhibition of isolated wheat chloroplasts on $K_{DT}$. O: no addition, ●: 1 μM DCMU, △: 1 μM DBMIB.
reinhardtii cells are photoinhibited in presence of atrazine the damage due to photoinhibition is significantly reduced.

The relationship between rate constants and $F_v/F_m$ ratio of control and photoinhibited chloroplasts is shown in Fig. 6.5. Although an increase in $K_{DT}$ causes a decrease in the efficiency of primary photochemistry, but it is not considered as photoinhibition damage as long as it is reversible (Demmig and Bjorkman 1987). Secondly, by definition itself, any increase in $K_{DT}$ does not reflect a disorder of the reaction center. The reduction in photochemistry, in this case, could be just because of the non-radiative decay or diversion of a fraction of the excitation energy in favor of PSI. The actual damage due to photoinhibition is reflected by a decrease in $K_P$ which results from inactivation of PSII reaction center complex. It does not seem to be rapidly reversible and recovery from photoinhibition requires synthesis of chloroplast encoded D1 protein (Ohad et al. 1984, Greer et al. 1986).

6.3.2 Electron transport studies

The results of fluorescence induction kinetics are further substantiated by a similar decrease in PSII mediated electron transport activity of chloroplasts. Photosystem II activity of chloroplasts, measured as $O_2$ evolution with
Fig. 6.5 Relationship between the rate constant for photosystem II photochemistry ($K_p$: open symbols) and non-radiative radiative energy dissipation and transfer to photosystem I complex ($K_{DT}$: filled symbols) and the ratio of variable to maximum in vivo chlorophyll a fluorescence ($F_v/F_m$) measured at room temperature.
silicomolybdate as the electron acceptor, was observed to decrease with increase in duration of photoinhibition. The damage to electron transport activity was reduced when chloroplasts were photoinhibited in presence of DCMU whereas the activity was reduced to a greater extent when DBMIB was present during illumination (Fig. 6.6). The data is consistent with our results of fluorescence induction kinetics which indicates that a protection is provided by DCMU to the chloroplasts from high light stress. On the contrary, DBMIB shows the ability to enhance the effect of high light stress on the chloroplasts.

6.3.3 Photo-oxidation of pigments

Photo-oxidation of chlorophylls on exposure of leaves of isolated chloroplasts to high photon flux densities for a long period has been reported by many workers (Kislyuk 1979, Ludlow 1987). Chlorophyll bleaching in our experiment did not exceed 10 percent during the first 20 min. of photoinhibition. Photobleaching of chlorophylls, however, increased to about 30 percent in the next 20 min. of photoinhibition (Table 6.2). Although photo-oxidation of chlorophylls might drastically affect the fluorescence characteristics of chloroplasts, but the observation that the extent of bleaching was not significantly different either in presence or absence of DCMU or DBMIB suggests that
Fig. 6.6 Electron transport activity of isolated wheat chloroplasts photoinhibited in absence (○) and presence of 1 μM DCMU (●) or 1 μM DBMIB (▲). Chloroplasts (100 μg Chl/ml) were preilluminated at 2250 W/m² at 25°C for 0 to 40 minutes before measuring oxygen evolution. The assay mixture contained 100 mM sucrose, 40 mM Hepes-KOH (pH 7.0) 15 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, 5 μM DCMU, 0.1 mM potassium ferricyanide, 0.5 mM silicomolybdate and chloroplast equivalent to 50 μg Chl/ml.
the observed differences in $F_v/F_m$ ratio and electron transport activities may not be caused by pigment loss. It may also be mentioned that the differences due to DCMU and DBMIB were apparent in the first 20 min. of photoinhibition when not more than 10 percent chlorophylls were degraded.

Under high light conditions leading to photoinhibition, there is an excess of dioxygen, and the plastoquinol population dominates over the oxidized quinones. In such a state, the $Q_B$ binding site on 32 kDa D1 protein remains unoccupied (because of low binding affinity of plastoquinol for the 32 kDa D1 protein) even though a reduced primary quinone ($Q_A^-$) is awaiting for transfer of its electron. Dioxygen, at this juncture, might accept electrons from $Q_A^-$ to form $O_2^-$ right inside the binding pocket which might itself cause a damage to the D1 protein. Increase in $O_2^-$ could also lead to the formation of more reactive species like hydroxyl free radicals, which might in turn, damage the D1 protein. This hypothesis was also supported by Asada and Takahashi (1987), who demonstrated a light induced generation of $O_2^-$ radicals within the lipid bilayers. The plausibility of semiquinone-mediated $O_2^-$ radical attack on the D1 protein finds more attention because of observation of accelerated turnover of the mitochondrial proteins which catalyze similar quinone mediated electron transfer
Table 6.2

Changes in the pigment content of isolated wheat chloroplasts on photoinhibition at 2250 $W/m^2$ at 25°C for 40 minutes. Light treatments were given in absence or presence of 1µM DCMU or 1µM DBMIB.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DCMU</th>
<th>DBMIB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chlorophyll a</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>100+1</td>
<td>100+1</td>
<td>100+1</td>
</tr>
<tr>
<td>20 min</td>
<td>91+2</td>
<td>90+0</td>
<td>91+1</td>
</tr>
<tr>
<td>40 min</td>
<td>71+5</td>
<td>70+4</td>
<td>78+5</td>
</tr>
<tr>
<td><strong>Chlorophyll b</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>28+1</td>
<td>30+1</td>
<td>28+1</td>
</tr>
<tr>
<td>20 min</td>
<td>28+0</td>
<td>28+0</td>
<td>26+1</td>
</tr>
<tr>
<td>40 min</td>
<td>27+1</td>
<td>27+1</td>
<td>26+1</td>
</tr>
<tr>
<td><strong>Chl a + Chl b</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>128+2</td>
<td>130+2</td>
<td>128+2</td>
</tr>
<tr>
<td>20 min</td>
<td>119+2</td>
<td>118+0</td>
<td>117+2</td>
</tr>
<tr>
<td>40 min</td>
<td>81+6</td>
<td>80+5</td>
<td>90+5</td>
</tr>
</tbody>
</table>
reactions. The auto-oxidation of semiquinone, which could also lead to the generation of superoxide and hydroxyl radicals, has also been shown to occur in sub-mitochondrial particles (Ksenzenko et al. 1983) and proteolysis in isolated mitochondria has been found to increase with the rate of electron flow (Dean and Pollak 1985).

Herbicides have been shown to compete with the quinones for binding site on the 32 kDa \( Q_B \)-protein (Pfister and Arntzen 1979). We have used DCMU during photoinhibitory treatment to provide a competitive inhibitor to the dioxygen for the \( Q_B \)-binding site. The decrease in the extent of photoinhibition observed in presence of DCMU suggests that high light induced damage to chloroplasts occurs at the \( Q_B \) binding site. One of the mechanisms by which DCMU may reduces the photodamage could be that it does not leave the \( Q_B \)-binding site unoccupied even at the time of overstimulation, thus reducing probability of formation of \( O_2^- \) in the \( Q_B \)-binding pocket of the 32 kDa Dl protein. To test this model, we have alternatively used DBMIB at the time of photoinhibitory treatment. DBMIB, at low concentrations, has been shown to selectively inhibit re-oxidation of PQH\(_2\) by the cyt \( b_6/f \) complex (Trebst 1980), thus increasing the concentration of reduced quinones which are thought to initiate the cascade of reactions leading to the formation of active species. Our results show an
enhancement of damage in presence of DBMIB. The results are in agreement with the reports on the decreased turnover rate of Dl protein in presence of herbicides (Mattoo et al. 1984).

Our results support the involvement of semiquinone anion radicals in the damage of Dl protein and indicate the protecting effect of the herbicide DCMU which could bind to the Dl protein. The results are further substantiated by enhancement of damage by the presence of DBMIB during photoinhibition. On the other hand, restoration of $F_v$ in presence of hydroxylamine suggest the acceptor side of PSII complex to be at least one of the targets. Thus although semiquinone anions are involved in the photoinhibition damage to chloroplasts but the damage is not only confined to the acceptor side of PSII. The results are in agreement with previous studies suggesting several different photoinduced lesions between Z and $Q_A$ have been proposed (Allakhverdiev et al. 1987, Callahan et al. 1986, Demeter et al. 1987, Styring et al. 1990). The mechanism of damage is still not clear and identification of the actual free radical species involved in photoinhibition damage to the Dl protein is in progress using a number of free radical scavengers specific to different free radicals. A quantitative estimation of the local population of free
radical species in the vicinity of $Q_B$-binding pocket would also be useful for a better understanding of the mechanism of high light induced damage to the D1 protein.