Chapter 4

STRESS-INDUCED ALTERATIONS IN THE STRUCTURE AND FUNCTIONS OF CHLOROPLASTS: EFFECTS OF HEAT AND HIGH LIGHT STRESS ON IN VIVO CHLOROPHYLL FLUORESCENCE CHARACTERISTICS AND PEROXIDATION OF THYLAKOID LIPIDS

Content

4.1 Introduction

4.2 Materials and Methods
   4.2.1 Recording of fluorescence transients
   4.2.2 Assay of lipid peroxidation

4.3 Results and Discussion
   4.3.1 In vivo chlorophyll fluorescence
      4.3.1.1 High temperature stress
      4.3.1.2 High light stress
   4.3.2 Lipid peroxidation in thylakoid membranes
      4.3.2.1 High light and temperature stress
   4.3.3 Interaction of high light and temperature stress
4.1 Introduction

Loss of photosynthetic activity in response to heat (Berry and Bjorkman 1980, Sayed et al. 1989, Brudvig et al. 1989, Thompson et al. 1989, Bilger and Schreiber 1990) and high light intensity (Powles 1984, Greer and Laing 1990, Styring et al. 1990, Telfer et al. 1990) has been reported by many workers. A concurrent loss in the efficiency of excitation energy transfer between the pigment-protein complexes of thylakoid membranes has also been observed which has been suggested to result from functional dissociation of the peripheral light harvesting chlorophyll-protein complexes from the reaction center of PSII (Schreiber and Armond 1978). The photosynthetic activity is reduced drastically on exposure of isolated chloroplasts to light intensities in excess of that required to saturate photosynthesis.

In vivo chlorophyll fluorescence has been used as an intrinsic probe for studying the photosynthetic efficiency of chloroplast membranes (van Kooten and Snel 1990, Demmig and Bjorkman 1987, Ogren and Oquist 1984, Schreiber and Berry 1977). It is known that high light treatment of the leaves or isolated chloroplasts leads to inactivation of PSII with little or no effect on PSI complex (Critchley and Smillie 1981, Powles and Bjorkman 1982). Photoinhibition has
been reported to interact with other accompanying environmental stresses (Greer et al. 1986). Environmental stresses affecting the rates of CO$_2$ fixation and photosynthetic electron transport, are suggested to enhance the photoinhibition damage to the chloroplasts.

An appreciable amount of work has been done on the characterization of the damage to thylakoid proteins due to environmental stresses but most of them have been concentrated on the damage to the functionally active protein components. Comparatively little attention has been paid to the alterations in the lipid components of the thylakoid membranes in response to environmental stresses. All the supramolecular pigment-protein complexes of chloroplast are embedded in lipid matrix which is significantly different from that of other membranes. One of the differences is the presence of highly unsaturated fatty acyl residues. The adaptation of thylakoid membrane to conserve such a distinct population of lipids must be in some way useful for the optimal functioning of the photosynthetic apparatus. Damage to the lipid components of thylakoid membranes is also likely to affect the photosynthetic activity of chloroplasts. In this chapter, we have attempted to examine the effect of high temperature, photoinhibition and their interaction on both the photo-
synthetic efficiency of isolated wheat chloroplasts and the nature of damage to the lipid components.

4.2 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 (Chapter 2). The chloroplasts were subjected to heat treatment or photoinhibition as described in Chapter 3.

4.2.1 Recording of fluorescence transients

Fluorescence induction curves were recorded at room temperature for 6 sec. on a transient recorder (TRl, Hansatech, England) after keeping the samples in complete darkness for 30 min. The samples were excited with blue actinic light using a Corning CS 4-96 glass filter between the sample and lamp. Emission kinetics was recorded through an interference filter (Wratten 89B). The rate constants for PSII photochemistry ($K_P$), non-radiative energy dissipation and energy transfer to PSI ($K_{DT}$) and energy dissipation as fluorescence ($K_F$) were calculated according to Bjorkman (1987). The rate constants for non-radiative energy dissipation in the pigment bed ($K_D$) and for energy transfer to PSI ($K_T$) were merged into a single rate constant.
\( K_{DT} = K_D + K_T \). The assumptions made for the calculations were that the unstressed leaves show the ratio of \( F_v / F_m \) equal to 0.864. \( K_P \) was assumed to be constant and its value was set to 1 (Bjorkman 1987). Thus in unstressed chloroplasts the relative values of the rate constants were \( K_P = 7K_{DT} = 70K_P \). The value of \( F_m \) in unstressed chloroplasts was set at 100 and all other readings were normalized accordingly.

4.2.2 Assay of lipid peroxidation

Peroxidation of thylakoid lipids after exposure of chloroplasts to different temperatures or light intensities was determined spectrophotometrically by formation of MDA as described in Chapter 2. Time course of lipid peroxidation was determined by exposing the chloroplasts to desired PFDs or temperatures for 0 to 20 min. Lipid peroxidation was also measured after photoinhibition of chloroplasts in presence of 1,4-diazabicyclo-[2.2.2]octane (DABCO) and phenazine methosulphate (PMS). DABCO and PMS were used as quenchers of singlet oxygen and superoxide free radical respectively.

4.3 Results and Discussion

4.3.1 In vivo chlorophyll fluorescence

In vivo chlorophyll fluorescence induction has been used for long as a non-invasive technique for the investigation of
stress-induced alterations in higher plant chloroplasts (Bilger and Schreiber 1990, van Kooten and Snel 1990). The ratio of variable to maximum chlorophyll fluorescence ($F_v/F_m$) is suggested to be a quantitative measure of the photochemical efficiency of photosystem II (Kitajima and Butler 1975). $F_v/F_m$ ratio is a quantitative indicator of reductions in the photon yield of $O_2$ evolution from intact leaves when exposed to environmental stress (Bjorkman and Demmig 1987, Demmig and Bjorkman 1987). A good correlation has also been shown to exist between the photon yield of photosynthesis and $F_v/F_m$ from PSII measured at ambient temperature (Demmig et al. 1987, Adams III et al. 1990).

4.3.1.1 High temperature stress

The $F_v/F_m$ ratio of unstressed chloroplasts, measured at room temperature, decreased with increase in the temperature of heat treatment. The decrease in $F_v/F_m$ ratio (Fig. 4.1a) and variable chlorophyll fluorescence (Fig. 4.1b) was slow up to 35°C beyond which it decreased sharply indicating a reduction in the photon yield of photosynthesis in wheat chloroplasts. The decrease in variable fluorescence and $F_v/F_m$ was, however, a result of two events occurring simultaneously: (i) the decrease in the level of $F_m$, and (ii) an increase in $F_o$ (Fig. 4.1b).
The intrinsic chlorophyll fluorescence, $F_0$, is suggested to be the level of fluorescence emission when all PSII reaction centers are open, i.e. all the primary quinone acceptors $Q_A$ are in oxidized state (Cao and Govindjee 1990). This would mean that $F_0$ is the emission from light harvesting complex before the transfer of excitation energy to the reaction center complex (see Papageorgiu 1975). If the LHCII is well connected to the reaction center and the transfer of excitation energy is efficient, the level of $F_0$ is expected to be low because most of the light energy absorbed by the LHC would be transferred to the reaction centers. A rise in $F_0$ may indicate dissipation of absorbed energy through the non-productive means of fluorescence emission rather than transfer to the reaction center and utilization in the photochemistry. Thus the increase in $F_0$ in our experiments may account for a less efficient transfer of absorbed energy to the reaction center which could be a consequence of stress induced dissociation of LHC from the reaction center. Apart from being transferred to the reaction center and used for chemical work, a number of possibilities exist for de-excitation of the absorbed light energy. It may be transferred to PSI complex or dissipated as heat or fluorescence emission without being conserved as chemical energy. The rate constants for dissipation of excitation energy as heat and transfer to PSI complex ($K_{DT}$)
Fig. 4.1 $F_v/F_m$ ratio (a), $F_v$, $F_m$ and $F_v$ (b) and $K_p$ and $K DT$ (c) of isolated wheat chloroplasts after treating them to different temperatures for 1 min. each in dark. Panel (d) shows the relationship between the rate constants ($K_p$ and $K DT$) and $F_v/F_m$ ratio after heat treatment.
and the primary photochemistry of PSII (K_p) are plotted in Fig. 4.1c. The results suggest a decrease in the rate of photochemistry which might arise due to inhibition of the reaction center functions and an increase in K_{DT} which may indicate a loss of absorbed light energy as heat or transfer of absorbed energy preferentially to PSI complex. This increase in K_{DT} could be explained by the disruption of contact between LHC and reaction center of PSII. The relationship between rate constants K_p and K_{DT} and F_v/F_m is shown in Fig. 4.1d.

4.3.1.2 High light stress

The results of experiments on the effect of high light intensity on isolated wheat chloroplasts are depicted in Fig. 4.2. There was a reduction in F_v/F_m ratio of the chloroplasts after being illuminated at different PFDs (Fig. 4.2a). The difference from the previous case is that the rate of photosynthetic efficiency decreases linearly with increase in light intensity whereas in case of heat treatment it decreased slowly up to 35°C beyond which the decrease was relatively sharp. The behavior of F_o', F_m and F_v with increase in light intensity are illustrated in Fig. 4.2b. There was a reduction in variable fluorescence with increase in the intensity of light treatment. The decrease in F_v was caused by a reduction in F_m and an increase in F_o.
Adir et al. (1990) have suggested that light induced changes in PSII complex of chloroplasts could be reversible as well as irreversible in nature. The reversible change, in green algae, has been suggested to result from a destabilization of the semiquinone anion $Q_B^-$ causing an increase in the intrinsic chlorophyll fluorescence ($F_O$). Ohad et al. (1988) have provided evidence for a light induced reversible conformational change of PSII reaction center affecting the $Q_B$ binding site located within the D1 protein. It has been concluded that the conformational change of PSII reaction center does not alter the primary photochemistry of charge separation (Adir et al. 1990). The reduction of $P680^+$ has also been reported to slow down considerably due to an inactivation of $Z$ which is identified as tyrosine-161 of the D1 protein (Debus et al. 1988). The persisting $P680^+$ might act as a fluorescence quencher (Itho et al. 1984) which may explain the lowering of maximal chlorophyll fluorescence observed under photoinhibitory conditions (Ohad et al. 1988, Kyle 1987). Thus the loss of $F_V$ and $F_V/F_m$, observed in our experiments may be explained by the $Q_B$-destabilization and slower rate of $P680^+$ reduction.

The following irreversible phase of photoinhibition is associated with the degradation of the D1 protein which is a component of the PSII reaction center complex. A decrease in
Fig. 4.2 $F_v/F_m$ ratio (a), $F_v$, $F_m$, and $F_v$ (b) and $K_P$ and $K_{DT}$ (c) of isolated wheat chloroplasts after exposing them to different light intensities for 1 min. each at 25°C. Panel (d) shows the relationship between the rate constants ($K_P$ and $K_{DT}$) and $F_v/F_m$ ratio after photoinhibition.
KP and an increase in $K_{DT}$ observed in case of light treatment of chloroplasts (Fig. 4.2c) suggests an inhibition of the rate of primary photochemistry of PSII and loss of excitation energy through non-productive means. The behaviour of $K_P$ and $K_{DT}$ with respect to $F_v/F_m$ on light treatment of chloroplasts are plotted in Fig. 4.2d. The actual estimate of photoinhibition is reflected by the decrease in $K_P$ because it represents the rate of photochemistry of the PSII complex, whereas the increase in $K_{DT}$ may also result by physical means without damaging the function of any component of the thylakoid membrane.

4.3.2 Lipid peroxidation in thylakoid membranes

Protein components of the thylakoid membrane have been suggested as the major target of damage induced by environmental stresses like high light (Mattoo et al. 1984, Ohad et al. 1988) and high temperature (Volger and Santarius 1981, Brudvig et al. 1989, Thompson et al. 1989). A great deal of attention has been paid to the molecular structure and organization of light harvesting antenna complex and reaction center in thylakoid membranes (Murphy 1986, Ort 1986). It is now becoming apparent that the unusual lipid composition of thylakoid membrane not only fulfills a structural role in membrane organization, but may also play a functional role in the activity of several integral
membrane protein complexes (Pick et al. 1984, Siegenthaler et al. 1987, Horvath et al. 1987, 1989, Van Grup et al. 1988). The galactolipid, monogalactosyldiacylglycerol (MGDG), which does not form a lamellar structure in an aqueous system is present in thylakoid membrane in considerable amount and is suggested to be involved in protein packaging (Gounaris et al. 1983). The maintenance of an appropriate balance between bilayer and non-bilayer forming lipids is supposed to be a major constraint on the temperature lability of thylakoid membranes (Gounaris et al. 1983). Studies on the model systems have indicated that the non-bilayer forming lipids have an increased tendency of phase separation at higher temperatures (De Kruijff et al. 1979, Sen et al. 1982). High temperature induced phase separation of thylakoid lipids which has been observed to be consistent with the reports of granal destacking of thylakoid membranes (Armond et al. 1980a, Staehelin 1986) could be a reason for the functional disruption of LHCP from the PSII core.

Chloroplasts are particularly prone to oxygen-toxicity effects. Firstly because their internal oxygen concentration in light is always greater than that in the surroundings, due to oxygen production in photosystem II complex. Secondly, the lipids present in chloroplast envelope and

81
thylakoid membranes contain a high percentage of poly-unsaturated fatty acids and are thus very susceptible to peroxidation. Sensitised chlorophylls have been suggested to catalyse the formation of singlet oxygen which could damage the thylakoid components (Heath and Packer 1968). Singlet oxygen is produced through photosensitized reactions, by oxidation of $\text{H}_2\text{O}_2$ and superoxide and from lipid peroxy radicals in chloroplasts (Russel 1957, Kanofsky and Axelrod 1986). Chlorophyll in its free state is an efficient photosensitizer, reducing triplet excited chlorophyll from the singlet excited state through intersystem crossing and then producing singlet oxygen through subsequent energy transfer from excited triplet chlorophyll to triplet dioxygen (Asada and Takahashi 1987). Triplet chlorophyll is formed in PSII and PSI reaction center complexes by radical pair recombination when the electron acceptor is reduced, depleted or inactivated after illumination. It has also been suggested that production of excited triplet chlorophyll and singlet oxygen is enhanced in chloroplasts under the conditions where the supply of electron acceptors is limited (Rutherford and Mullet 1981, Setif et al. 1981, Takahashi and Katoh 1984). The supply of electrons to the reaction center may be restricted due to a damage to oxygen evolving complex. Although singlet oxygen has not been directly detected in illuminated chloroplasts, possibly due to its
short lifetime, but the production of singlet oxygen in illuminated thylakoids has been deduced from the peroxidation of thylakoid lipids. The production of singlet oxygen is enhanced in D₂O in which it has an extended lifetime (Kearns 1979). Singlet oxygen attracts more attention because the lifetime of singlet oxygen in the hydrophobic interior of membranes is much greater than it is in aqueous solution. In addition to lipid peroxidation and the formation of reactive aldehydes by peroxide decomposition, hydrolysis of lipids also occurs which releases fatty acids. Both esterified and released fatty acids undergo peroxidation, and the free fatty acids themselves cause membrane damage and inhibit photosynthesis.

4.3.2.1 High light and temperature stress

Isolated chloroplasts, on illumination, are shown to produce substances which react with thiobarbituric acid (TBA) by Heath and Packer (1968). TBA reactive substances signify the formation of the hydroperoxides of the polyunsaturated fatty acids. Lipid peroxidation has been defined as oxidative deterioration of polyunsaturated lipids which contain more than two carbon-carbon double covalent bonds. In biological systems, the occurrence of peroxidation is usually correlated with malondialdehyde (MDA) production (Girotti 1990). MDA production in our experiments was observed to
Fig. 4.3 Peroxidation of thylakoid lipids on heat treatment of isolated chloroplasts for different periods from 0 to 20 min. in dark. ○: 30°C, ●: 40°C, △: 45°C, ▲: 50°C.
Fig. 4.4 Peroxidation of thylakoid lipids on photoinhibition of isolated chloroplasts for different periods from 0 to 20 min. at 25°C. ○: 190, ●: 590, △: 980, ▲: 1200 W/m².
increase linearly with increase in the period of heat (Fig. 4.3) and light treatment (Fig. 4.4). A comparison of Fig. 4.3 and 4.4 indicates that the peroxidation of fatty acyl residues of thylakoid membranes is more in response to light treatment as compared to heat treatment. A comparison of peroxidation of thylakoid lipids on heat and high light treatment shows that high light stress may cause greater extent of thylakoid lipid peroxidation compared to high temperature stress. The stress-induced peroxidation of thylakoid lipids might indicate an involvement of singlet oxygen. We have used a specific quencher of singlet oxygen DABCO (Takahama and Nishimura 1975) during photoinhibition of chloroplasts to investigate the role of singlet oxygen in photoinhibition. A substantial decrease in the extent of thylakoid lipid peroxidation was observed in presence of 20 mM DABCO which indicates involvement of singlet oxygen in the high light induced peroxidation of thylakoid lipids. Phenazine methosulfate which scavenges superoxide free radicals (Takahama and Nishimura 1979) also resulted in a reduction of lipid peroxidation (Fig. 4.5). On the basis of these results, it may be concluded that peroxidation of thylakoid lipids is mediated by the action of singlet oxygen and superoxide free radicals which are produced during illumination of chloroplasts at high PFD. However, it may be mentioned that singlet oxygen and superoxide free radicals
Fig. 4.5 Lipid peroxidation in chloroplasts in absence (○) and presence of 20 mM DABCO (●) or 20 μM PMS (△).
generated in the chloroplasts might also lead to production of other free radical species such as hydroxyl free radicals which may be potentially more toxic to the photosynthetic apparatus. Although the mechanism of generation of singlet oxygen in chloroplasts is not clear but a number of workers have suggested that under conditions of limiting electron transfer through the photosystems energy dissipation via charge separation in the photosystems may be decreased in favour of reactions involving intersystem crossing and thus enhancing the singlet oxygen formation in isolated chloroplasts as reviewed by Elstner (1980, 1982).

Greenberg et al. (1990) have provided evidence for the involvement of semiquinone anion radicals ($Q_-^B$) in the photoinhibition of Spirodea oligorhiza. Semiquinone anion radicals are formed in visible light due to its role as a two electron gate in PSII (Crofts and Wraight 1983), but this species alone has been suggested to be insufficient for degradation of the D1 protein under photoinhibitory conditions. Oxygen could be a powerful mediator of free radical reactions (Kyle 1987, Halliwell and Gutteridge 1989) and generate several free radical species which might be more effective than the semiquinone anion radicals in damaging the D1 protein as well as the polyunsaturated fatty acyl residues of the thylakoid lipids.
Fig. 4.6 Peroxidation of thylakoid lipids as a function of temperature of heat treatment (a) and PFD (b). Chloroplasts were exposed to different temperatures and light intensities for 10 min. each.
When the extent of lipid peroxidation was plotted against temperature, a break at 40°C was observed beyond which the rate of lipid peroxidation was significantly higher (Fig. 4.6a). No such break was observed in case of light treatment (Fig. 4.6b). The break at 40°C is interesting to note because there are reports suggesting a high temperature induced phase separation of thylakoid lipids. The fact that no such break was observed in case of light intensity curve suggests this notion because high light intensity, so far, has not been reported to cause a phase separation in vitro or in vivo. The other point which may be mentioned here is that this temperature coincides well with the critical temperature for irreversible heat-induced injury to the photosynthetic apparatus of wheat.

4.3.3 Interaction of high light and temperature stress

The interaction of heat and high light stress at the level of thylakoid membrane was studied by simultaneous and sequential imposition of the two stresses. Three different temperatures (40, 45 and 50°C) and light intensities (590, 1200 and 1750 W/m²) were chosen for the study. A change in the extent of reduction of variable fluorescence and $F_v/F_m$ was observed (Fig. 4.7) by mere change in the order of the two stresses. The reduction in $F_v$ and $F_v/F_m$ ratio was observed to be more when the chloroplasts were
Fig. 4.7 Effect of interaction of heat and high light stress on variable Chl fluorescence, $F_v$ (top) and $F_v/F_m$ ratio (bottom) of isolated wheat chloroplasts. High temperature and high light stresses were imposed simultaneously (blank bars) and sequentially, heat stress preceding photoinhibition (diagonal bars) and photoinhibition preceding high temperature treatment (filled bars).
subjected to light stress prior to heat treatment. The reduction in variable fluorescence and $F_v/F_m$ was also accompanied by an increase in the extent of peroxidation of fatty acyl residues of the thylakoid lipids (Fig. 4.8). It is also interesting to note that when high temperature and high light treatments were given simultaneously, the levels of $F_v/F_m$ and $F_v$ and the extent of lipid peroxidation was between the two sequential combinations of the above mentioned stresses (Fig. 4.7 and 4.8). It has been suggested that if photosynthetic electron transport is inhibited by any accompanying stress, the reaction center chlorophylls can not dispose their excitation energy, nor can they accept excitation energy from their harvesting pigment-protein complexes. The probability of transfer of electrons to oxygen present in the surrounding thus increases manifolds which may result in an increased generation of singlet oxygen. This hypothesis is further substantiated by our observations that the reduction in the variable chlorophyll fluorescence, $F_v/F_m'$, increase in lipid peroxidation and decrease in electron transport activity (as discussed in the Chapter 3) was more when the wheat chloroplasts were subjected to photoinhibition prior to heat treatment because of an increased rate of generation of singlet oxygen due to light treatment which is later manifested as the exacerbation of damage to the tissue by subsequent heat
Fig. 4.8 Effect of interaction of heat and high light stress on peroxidation of thylakoid lipids in isolated wheat chloroplasts. High temperature and high light stresses were imposed simultaneously (blank bars) and sequentially, heat stress preceding photoinhibition (diagonal bars) and photoinhibition preceding high temperature treatment (filled bars).
treatment. Conversely, heat treatment of chloroplasts is unlikely to produce singlet oxygen in an appreciable amount hence resulting in a lesser extent of damage to the chloroplasts.