Chapter 4

PHOTOINHIBITION OF INTACT WHEAT LEAVES: INVOLVEMENT OF FREE RADICAL SPECIES AND THE ROLE OF ENZYMATIC SCAVENGERS

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4.1 INTRODUCTION

Green plants possess the ability to collect light from the visible range of the solar spectrum (400-700 nm) and transform it efficiently into chemical energy. However, the absorption of excess light energy may cause damage to the photosynthetic apparatus and result in a decrease in the quantum yield of photosynthesis which is referred to as photoinhibition (Powles, 1984). The present evidences strongly suggest that the PSII complex of higher plants is highly susceptible to photoinhibition (Ohad et al., 1985; Arntz and Trebst, 1986; Virgin et al., 1988; Setlik et al., 1990). Photoinhibition in green plants results in photo-degradation of the D1 protein which is an intrinsic component of the PSII reaction center (Theg et al., 1986; Nanba and Satoh, 1987; Kuhn and Boger, 1990). The molecular mechanism of photoinactivation of PSII complex including the degradation of D1 protein still remains largely hypothetical and there are uncertainties regarding the primary site of damage within the PSII complex.

Oxygen which is evolved during photosynthesis in higher plants, has been shown to strongly influence photoinhibition (Elstner, 1982, 1987; Krause and Cornic, 1987; Asada and Takahashi, 1987). Several studies have suggested a role of
active oxygen species, which under strong illumination may be produced at rates exceeding the capacity of scavenging system of the leaves (Asada and Takahashi, 1987; Hodgson and Raison, 1991). Dioxygen can be activated in higher plant chloroplasts in a number of ways to produce reactive species like superoxide radical anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl free radicals (OH$^-$) and singlet oxygen (1O$_2$) (Elstner, 1987). Active oxygen species are toxic to cells and cause non-specific oxidation of macromolecules resulting in their degradation.

Chloroplasts possess scavenging system which protects the photosynthetic apparatus efficiently from the damaging reactions of active oxygen particularly at normal light intensities (Schoner and Krause, 1990; Asada and Takahashi, 1987). The active oxygen scavenging system in plants consists of several enzymes such as superoxide dismutase (SOD), ascorbate peroxidase, catalase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase (Larson, 1988; Schoner and Krause, 1990). However, when the excitation energy is in excess of the energy utilized in photosynthesis, rate of oxygen activation may increase and enhance photoinhibition.

It has been suggested that the process of recovery is operational through the synthesis of damaged D1 protein
concomitant with the exposure of leaves to photoinhibitory conditions (Ogren et al., 1984; Samuelsson et al., 1985; Lidholm et al., 1987). Photoinhibition damage appears only when the rate of photodegradation of D1 protein exceeds the rate of its repair (Ludlow, 1987). An effort was made to investigate the synthesis of the proteins damaged during photoinhibition by using CHI and CAP which are known to inhibit the nuclear and chloroplast directed protein synthesis respectively (Kyle et al., 1984; Greer et al., 1986).

Various environmental stress factors, e.g. chilling, heat and drought can induce photoinhibition of photosynthesis even at moderate light intensities (Kyle, 1987, Somersalo and Krause, 1988, 1989). An increased susceptibility to light stress at low temperatures may result from restricted carbon metabolism, which predisposes the photosynthetic apparatus to over-energization and promote the formation of active oxygen species. We have studied the involvement of active oxygen species during photoinhibition by monitoring the changes in the activities of antioxidant enzymes which are able to detoxify the activated oxygen species. The temperature dependence of photoinhibition of photosynthesis was also studied by assaying the electron transport activity and measuring the chlorophyll fluorescence kinetics. Peroxidation of thylakoid lipids was also
estimated in order to examine the role of reduced species of oxygen.

4.2 RESULTS AND DISCUSSION

4.2.1 Electron transport activity

The effect of strong visible light on the photosynthetic activity was studied by measurement of electron transport activity in chloroplasts isolated from leaves photoinhibited for different periods of time up to 10h. The intensity of photoinhibitory light was 600 W.m\(^{-2}\) and the treatments were given at 25 and 8\(^{\circ}\)C to study the effect of temperature on photoinhibition of photosynthesis.

The time course of DCIP photoreduction in chloroplasts isolated from wheat leaves exposed to a PFD of 600 W.m\(^{-2}\) at 25\(^{\circ}\)C in the presence of CHI and CAP are shown in Fig. 4.1a. It is clear from the diagram that inhibition of electron transport activity (H\(_2\)O to DCIP) was reduced more rapidly in the presence of protein synthesis inhibitors. There was a 35% decline in the electron transport activity when the leaves were exposed to a PFD of 600 W.m\(^{-2}\) at 25\(^{\circ}\)C for 10h in the absence CHI and CAP (Fig. 4.1a). This decrease in electron transport activity may be attributed to photoinhibitory damage to the primary photochemical reactions of PSII. Light treatment of leaves to PFD of 600 W.m\(^{-2}\) at 25\(^{\circ}\)C for 10h in
presence of CHI resulted in 49% decrease in the rate of DCIP photoreduction (Fig. 4.1a). The presence of CAP, a chloroplast protein synthesis inhibitor, during exposure to high light stress caused a 63% inhibition in the electron transport activity (Fig. 4.1a). The D1 protein which plays an important role in PSII photochemistry is encoded by the chloroplast genome. The synthesis of this protein is likely to be inhibited in CAP treated plants and the plants might fail to replace the photodegraded D1 protein which may result in an inhibition of electron transport activity. D1 protein is known to have a high turnover rate in light and photoinhibition of intact leaves results in its photodegradation (Kyle, 1985; Mattoo et al., 1984, 1989).

Our results show that both the nuclear and chloroplast directed protein synthesis, play important role in the synthesis and replacement of the photodamaged PSII proteins during high light stress. However, the results show that inhibition of chloroplast directed protein synthesis resulted in greater damage to photosynthetic electron transport chain as compared to nuclear directed protein synthesis. These results are also in accordance with the hypothesis that the degree of photoinhibition is a balance between the processes of repair and damage (Skogen et al., 1986; Greer et al., 1986). These results are in agreement with the
Photosynthetic electron transport activity as a function of period of light treatment in absence and presence of protein synthesis inhibitors. Open circles: no addition, filled circles: 10 μg.ml⁻¹ CHI, open triangles: 200 μg.ml⁻¹ CAP. The seedlings were kept in CHI and CAP solutions for 12h prior to photoinhibition treatment. Results are expressed as percent of initial value. Wheat leaves were exposed to a photoinhibitory light of 600 W.m⁻² at 25°C (panel a) and 8°C (Panel b). Initial rates of DCIP photoreduction were 219, 158 and 119 μmol/mg Chl/h in untreated, CHI and CAP treated leaves respectively. Each point represent mean of three separate experiments.
reports of several authors who have demonstrated similar effects of inhibition of protein synthesis during photo-inhibition of algal cells (Setlikova et al., 1984; Ohad et al., 1984; Samuelsson et al., 1985, 1987).

High light stress at low temperature may cause changes in thylakoid membrane stability, resulting in reduced efficiencies of all membrane-bound photosynthetic processes (Graham and Patterson, 1982; Baker et al., 1983; Hodgson et al., 1987; Smillie et al., 1988). The effect of low temperature during high light treatment on the rate of Photosynthetic electron transport is shown in Fig. 4.1b. When leaves were photoinhibited with a PFD of 600 W.m$^{-2}$ at 8$^\circ$C, the electron transport activity was inhibited by 51% of the initial level after 10h (Fig. 4.1b). This inhibition of activity was light-dependent, since chilling in the dark produced no significant change in electron transport activity (data not shown). The exacerbation of photoinhibition damage at low temperature could be because of reduced rates of protein synthesis (Oquist et al., 1987), inhibition of alternative ways of dissipation of excitation energy (Hodgson and Raison, 1989) and generation of active oxygen species (Schoner et al., 1990). Several workers who have shown that exposure of plants to chilling temperatures in the range of 0 to 12$^\circ$C in light is much more damaging to
photosynthesis than in the dark (Lyons, 1973; Oquist, 1983; Ogren et al., 1984; Kee et al., 1986; Yakir et al., 1986).

The presence of CAP during light treatment resulted in higher susceptibility of wheat leaves to photoinhibition. The electron transport activity was reduced by 91% in CAP treated leaves photoinhibited at 8°C for 10h at 600 W m⁻² (Fig. 4.1b). This result shows that the plants rely on continuous chloroplast protein synthesis for retention of photosynthetic competence (Lidholm et al., 1987). Ohad et al. (1984) have proposed that the synthesis of the D1 protein is specifically required for the repair of photoinhibition damage in *Chlamydomonas*. However, the identity of specific protein damaged and synthesized during photoinhibition is not clear from our results.

Cycloheximide, which blocked cytoplasmic protein synthesis, enhanced the photoinhibition damage at 8°C as compared to 25°C. Presence of CHI during photoinhibition led to 81% reduction in the rate of electron transport. The reason for this is not known, however, it may be due to the prevention of alternative ways of dissipation of excess excitation energy during photoinhibition at lower temperatures (Oquist et al., 1987). The excess excitation energy may lead to the generation of active oxygen species which apart from the D1 protein, may destroy the nuclear encoded proteins also.
Huse and Nilsen (1989) have reported that addition of CHI during recovery inhibited the synthesis of several unidentified proteins and almost stopped synthesis of the LHC. CHI may perform the same function during photo-inhibition and cause a reduction in the photosynthetic electron transport activity. Lidholm et al. (1987) have also demonstrated a similar kind of effect in *Chlamydomonas reinhardtii* and suggested the occurrence of another type of photodamage which is supported by the results of Wettern and Ohad (1984).

Similar results were obtained for the DPC to DCIP electron transport activity in the chloroplasts isolated from untreated and CHI and CAP treated leaves after photoinhibition at 25 and 8°C (Fig. 4.2a and 4.2b). DPC is known to donate electrons to PSII bypassing the oxygen evolving complex. The ineffectiveness of DPC in the restoration of electron transport activity rules out any damage on the oxidizing side of PSII complex in wheat leaves. These results are in accordance with the reports suggesting the localization of the photoinhibition damage on the acceptor side of PSII (Cleland and Critchley, 1985; Arntz and Trebst, 1986; Cleland et al., 1986; Cleland and Melis, 1987).

### 4.2.2 *In vivo* chlorophyll fluorescence

*In vivo* chlorophyll fluorescence characteristics have been
Fig. 4.2 Photosynthetic electron transport activity measured in presence of 1mM DPC as a function of period of light treatment in absence and presence of protein synthesis inhibitors. Open circles: no addition, filled circles: 10 µg.ml⁻¹ CHI, open triangles: 200 µg.ml⁻¹ CAP. The seedlings were kept in CHI and CAP solutions for 12h prior to photoinhibition treatment. Results are expressed as percent of initial value. Wheat leaves were exposed to a photoinhibitory light of 600 W.m⁻² at 25°C (panel a) and 8°C (Panel b). Initial rates of DCIP photoreduction were 248, 165 and 122 µmol per mg Chl per hour in untreated, CHI and CAP treated leaves respectively. Each point represents mean of three separate experiments.
used to diagnose the effects of high light stress on intact higher plant leaves (Kitajima and Butler, 1975; Baker and Horton, 1987; Baker et al., 1989). The kinetics of chlorophyll fluorescence is associated not only with the photochemical activities of thylakoids but also with the metabolic processes related to carbon assimilation (Krause and Weis, 1984; Horton, 1985; Sivak and Walker, 1985; Horton and Hague, 1988). The induction curves for unstressed leaves and for leaves photoinhibited for 10h with 600 W.m\(^{-2}\) at 25\(^{\circ}\)C are shown in Fig. 4.3a. Unstressed leaves gave rise to a two-peaked curve typical of those reported previously for higher plant leaves (Baker and Bradbury, 1981; Briantais et al., 1986; Adams III et al., 1990). Exposure of leaves to 600 W.m\(^{-2}\) white light for 10h at 25\(^{\circ}\)C resulted in a significant decline in F\(_p\) (Fig. 4.3a) which suggests a reduction in the efficiency of primary photochemistry of PSII complex in the leaves. The M peak which is indicative of rate of CO\(_2\) fixation in the leaves also disappeared on photoinhibition.

Chlorophyll fluorescence characteristics of the leaves in which nuclear and chloroplast directed protein synthesis was inhibited by CHI and CAP respectively were recorded for investigation of the effects of protein synthesis in primary photochemical processes during photoinhibition. There was a significant decrease in F\(_p\) in unstressed leaves treated with CHI and CAP for 12h. However, the reduction in F\(_p\) was rela-
tively more in CAP treated plants (Fig. 4.3b and 4.3c). Photoinhibition of leaves of CAP treated plants resulted in a much greater extent of reduction in $F_p$ and the peak $P$ was completely lost on photoinhibition. Treatment of the plants with CHI and CAP for 12h also caused a marked reduction in the M peak which was completely lost after photoinhibition.

The reduction in chlorophyll fluorescence parameters of leaves of CHI and CAP treated plants even in the absence of high light stress indicates a requirement of protein synthesis during normal growth. The higher extent of reduction in Chl fluorescence in presence of CAP may be because of the requirement of chloroplast encoded proteins. D1 protein might be one of the important components as its synthesis is important for the plants to match with its high turnover rate in light (Greenberg et al., 1987). The decrease in primary photochemistry in the leaves of CAP treated plants could be due to the inhibition of D1 protein synthesis which has a functional importance being a constituent of PSII reaction center. Photoinhibition of the leaves in which plastid directed protein synthesis is not operational is likely to result in the failure of D1 protein synthesis which is known to be photodegraded at a faster rate during high light stress (Greenberg et al., 1989). This is reflected by our observation that the reduction in Chl fluorescence...
Fig. 4.3 The effect of photoinhibition of intact wheat leaves on the in vivo chlorophyll fluorescence transients in absence (Panel a) and presence of CHI (Panel b) and CAP (Panel c). The leaves were photoinhibited with 600 W.m\(^{-2}\) white light at 25°C for 10h. The seedlings were kept in CHI and CAP solutions for 12h prior to photoinhibition treatment. Curve 1 and 2 represent the fluorescence transients of unstressed and photoinhibited leaves respectively.
scence parameters was the maximal on photoinhibition of leaves in presence of CAP. These results also indicate that proteins are synthesized even during photoinhibitory treatment which supports the previous notion that synthesis or repair of the damaged proteins also goes on simultaneously with high light treatment of leaves (Kyle et al., 1984). The photoinhibition damage appears only when the rate of photo-degradation of the D1 protein is exceeded by the rate of its repair (Skogen et al., 1986; Greer et al., 1986).

Similar results were obtained for photoinhibition of CHI and CAP treated wheat leaves at 8°C. The effect of chilling (at 8°C) during photoinhibition was only qualitative as the extent of inhibition of Chl fluorescence parameters were higher (Fig. 4.4a, 4.4b and 4.4c) as compared to photoinhibition at 25°C. These effects are attributed to low temperature induced photoinhibition of photosynthesis in higher plants (Ogren and Oquist, 1984; Oquist et al., 1987). It is evident that photoinhibition of PSII is an important contributing factor limiting the quantum yield of carbon assimilation in wheat leaves chilled at high light (Ortiz-Lopez et al., 1990).

4.2.3 Chlorophylls

Exposure of wheat leaves to 600 W.m⁻² white light for 20h at
The effect of photoinhibition of intact wheat leaves on the in vivo chlorophyll fluorescence transients in absence (Panel a) and presence of CHI (Panel b) and CAP (Panel c). The leaves were photoinhibited with 600 W.m$^{-2}$ white light at 8°C for 10h. The seedlings were kept in CHI and CAP solutions for 12h prior to photoinhibition treatment. Curve 1 and 2 represent the fluorescence transients of unstressed and photoinhibited leaves respectively.
25 and 8°C did not result in any significant change in chlorophyll content in the leaf extracts (Table 4.1). Photoinhibition of photosynthesis and photo-oxidation of chlorophylls are known to be two different phenomena (Kyle, 1987). Photoinhibition may occur without any detectable changes in bulk leaf chlorophyll concentration (Powles, 1982; Bjorkman and Powles, 1984). Chlorophyll bleaching is observed only if the excess light is severe or prolonged (Bjorkman and Powles, 1984). The activities of all the scavenger enzymes are expressed on the basis of chlorophyll since the chlorophyll content of leaves gives a measure of potential absorption of excess light energy (Schoner et al., 1990).

4.2.4 Enzyme activities

Chloroplasts are exposed to high levels of oxygen due to oxygen evolution in PSII of higher plants. Products of oxygen reduction can lead to free radical mediated reactions in the cells with toxic consequences (Wheeler et al., 1990). The presence of active oxygen generating systems in the chloroplasts, together with high percentage of polyunsaturated fatty acids in the thylakoids, make chloroplasts susceptible to oxidative injury (Halliwell, 1984). Aerobic organisms possess an antioxidant defense system, which comprises of both enzymatic and non-enzymatic substances for scavenging free radicals. Depending upon the efficiency of
Table 4.1 Changes in the total chlorophyll content in wheat leaf homogenates extracted from photoinhibited leaves at 25 and 8°C with 600 W.m\(^{-2}\) white light.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Chl content (mg/ml leaf extract)</th>
<th>25°C</th>
<th>8°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40.3±0.1</td>
<td>43.3±1.3</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>39.8±0.9</td>
<td>43.0±0.8</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>39.3±0.4</td>
<td>42.1±0.7</td>
<td></td>
</tr>
</tbody>
</table>
the antioxidant defense systems, plants may differ in resistance to activated oxygen.

Oxygen toxicity may arise either by uncontrolled production or inefficient scavenging of active oxygen species. Under normal circumstances the levels of reduced oxygen products are low and they are effectively removed by the natural defense mechanisms of the cells. The concentration of active oxygen is likely to increase under stress conditions either due to increased production or lower scavenging capacity. These active oxygen species would attack the target molecules enhancing the photoinhibition damage (Reisman and Ohad, 1986). The activities of several enzymes responsible for scavenging of superoxide anion radicals and \( \text{H}_2\text{O}_2 \) were examined in leaf extracts in relation to photoinhibition at 25 and 8°C. The ascorbate content was also determined as it takes part in the scavenging of active oxygen species.

4.2.4.1 Catalase

Catalase functions in the decomposition of hydrogen peroxide which is produced by several \( \text{H}_2\text{O}_2^- \)-generating oxidases localized within the peroxisomes of higher plant leaves (Tolbert, 1971). Photoinhibition of wheat leaves at 25 and 8°C resulted in a decrease in catalase activity in the leaf
extracts (Fig. 4.5). The loss of catalase activity was greater when photoinhibition was performed at 8°C. Exposure of the leaves to 600 W.m\(^{-2}\) for 20h at 25°C resulted in a 37% decrease in catalase activity (Fig. 4.5a), whereas activity was inhibited by 72% after the same treatment at 8°C (Fig. 4.5b). No significant change in catalase activity was observed in extracts of the leaves kept in the dark at 25 and 8°C for 20h. These results suggest that the inactivation of catalase observed in our experiments may be mediated by light. Catalase is known to be a photolabile enzyme and its photo-oxidative degradation was first reported by Eyster (1950). Cheng et al. (1981) have shown that photoinactivation of catalase is mediated through light absorption by both the enzyme bound heme groups and chloroplast pigments and it depended on the presence of oxygen. The prosthetic heme groups are suggested to dissociate from the enzyme during its photoinactivation (Cheng et al., 1981). This inactivation has been suggested to be caused by the active oxygen species and organic peroxides such as the products of lipid peroxidation (Feierabend and Engel, 1986; Elstner, 1982; Takahashi and Asada, 1983). The enzyme which is photo-degraded under in vivo conditions is partly substituted by new synthesis. However, at lower temperatures, when the rate of protein synthesis is retarded the plants may not cope up
Fig. 4.5 Changes in catalase activity in leaf extracts prepared from unstressed and photoinhibited leaves with PFD of 600 W.m\(^{-2}\) at 25\(^{\circ}\)C (a) and 8\(^{\circ}\)C (b) for different periods of time. Open circles: no addition, filled circles: 10 µg.ml\(^{-1}\) CHI, open triangles: 200 µg.ml\(^{-1}\) CAP. The seedlings were kept in CHI and CAP solutions for 12h prior to photoinhibition treatment. Results are expressed in folds of the initial level. Each point represents the mean of three separate experiments.
with the fast photo-degradation of catalase resulting in a greater loss of activity.

The loss of catalase activity was pronounced in presence of CHI at both 25 and 8°C (Fig. 4.5a and 4.5b). Presence of CAP during photoinhibition did not result in any significant loss of catalase activity at both the temperatures under the study. The changes in the catalase activity on photoinhibition of CAP treated leaves was similar to those observed in the absence of any protein inhibitor (Fig. 4.5a and 4.5b). Our results suggest that photoinactivation of catalase occurs in leaves under high light intensity, however, it may not be observed under normal physiological conditions because of its compensation by nuclear directed de novo synthesis of the enzyme (Feierabend and Engel, 1986). In the presence of CHI, the loss of catalase activity was greater which indicates that synthesis of new catalase is blocked by the inhibition of the nuclear directed protein synthesis. The observation that the activity on photoinhibition of the leaves in presence of CAP followed a pattern similar to that when no inhibitor was present suggests that the inhibition of chloroplast directed protein synthesis had no effect on the activity of catalase. Hence it is evident that this enzyme is encoded by the nuclear genome.
4.2.4.2 Superoxide dismutase

SOD catalyzes the dismutation of superoxide anion radicals to hydrogen peroxide and oxygen (McCord and Fridovich, 1969. Monk et al., 1989). SOD in plants has been shown to be related with the protection from oxidative injury induced by environmental stresses (Burke et al., 1985; Dhindsa and Matowe, 1981). A gradual increase in SOD activity was observed in the extracts of leaves photoinhibited at 25°C with 600 W.m⁻² white light (Fig. 4.6a). SOD activity increased by about 1.55 folds after photoinhibition of the leaves for 20h in which protein synthesis was not inhibited (Fig. 4.6a). The increase in SOD activity might signify the production of superoxide anion radicals during photoinhibition of the leaves (Hodgson and Raison, 1991). Restricted carbon metabolism during photoinhibition leads to a depletion of the natural electron acceptor NADP⁺, thereby promoting the generation of superoxide via ferredoxin auto-oxidation (Badger, 1985; Asada and Takahashi, 1987). The increase in SOD activity under conditions of increased superoxide radical generation in higher plants and cyanobacteria has been reported by several workers (Hassan and Fridovich, 1977; Abeliovich et al., 1974; Rabinowitch and Fridovich, 1985; Tanaka and Sugahara, 1980).

Photoinhibition of leaves in which chloroplast directed
protein synthesis was inhibited by CAP did not result in any significant change in SOD activity (Fig. 4.6a). However, inhibition of nuclear directed protein synthesis by CHI resulted in a marked decrease in SOD activity (Fig. 4.6a). Inhibition of the increase in SOD activity on photoinhibition of CHI treated leaves may indicate that the increase in SOD activity was not only dependent on the amount of $O_2^-$ produced during photoinhibition but also on the rate of synthesis of new enzyme. These results also suggest that SOD is encoded by the nuclear genome.

Photoinhibition of leaves at $8^\circ$C resulted in a much faster increase in SOD activity as compared to photoinhibition at $25^\circ$C (Fig. 4.6b). 20h light treatment of leaves at 600 W.m$^{-2}$ at $8^\circ$C caused a 2.9 fold increase in SOD activity (Fig. 4.6b) whereas the same treatment at $25^\circ$C resulted in only 1.55 fold increase. These results are in agreement with that of Schoner and Krause (1990) who have reported an increase in SOD activity in cold-acclimated spinach leaves. The increase in SOD activity could be because of higher rates of superoxide generation during light treatment at $8^\circ$C. Several workers have suggested that generation of active oxygen species increases manifolds during photoinhibition of higher plants at low temperatures (Wise and Naylor, 1987; Hodgson and Raison, 1991). Similar to photoinhibition treatment at $25^\circ$C, the increase in SOD activity was retarded significan-
Fig. 4.6 Changes in SOD activity in leaf extracts prepared from unstressed and photoinhibited leaves with PFD of 600 W.m\(^{-2}\) at 25°C (a) and 8°C (b) for different periods of time. Open circles: no addition, filled circles: 10 µg.ml\(^{-1}\) CHI, open triangles: 200 µg.ml\(^{-1}\) CAP. The seedlings were kept in CHI and CAP solutions for 12h prior to photoinhibition treatment. Results are expressed in folds of the initial level. Each point represent mean of three separate experiments.
tly in the leaves devoid of nuclear directed protein synthesis (Fig. 4.6b). The plastid directed protein synthesis inhibitor CAP did not have any significant effect at 8°C also and the pattern followed closed to that in which the protein synthesis was not inhibited (Fig. 4.6b).

On the basis of these results it may be concluded that the SOD activity increases during photoinhibition of intact wheat leaves. The increase in activity is greater on photoinhibition at 8°C which may be due to higher rates of production of superoxide anion radicals. The activity of SOD during photoinhibition of intact wheat leaves is regulated by the concentration of its substrate superoxide radicals. Increase in superoxide may trigger the synthesis of new enzyme as the amount of SOD present under normal conditions may not be able to cope up with higher concentrations of the active species produced during photoinhibition. However, the mechanism by which the synthesis of SOD is triggered remains to be established. The increase in SOD activity may be a manifestation of the defense system which may provide stability to the photosynthetic apparatus against photoinhibition damage by scavenging the toxic superoxide anion radicals (Clare et al., 1984).
4.2.4.3 Ascorbate

Control plants showed an average ascorbate content of 2.1 μmol.mg Chl\(^{-1}\) which is comparable to the published data (Foyer et al., 1983; Gillham and Dodge, 1986). Ascorbate content increased by about 1.2 folds after 20h exposure of the leaves to 650 W.m\(^{-2}\) at 25°C (Fig. 4.7a). The same treatment at 8°C resulted in a 1.8 fold increase in ascorbate content (Fig. 4.7b). This increase in ascorbate content may be due to higher rates of ascorbate synthesis in light (Hossain and Asada, 1984). Ascorbate in chloroplasts serves as a potent reducing agent (Levitt, 1980; Chinoy, 1984), and together with glutathione, it is involved in enzymatic and non-enzymatic detoxification of the active oxygen species (Asada and Takahashi, 1987; Elstner, 1987). Thus increase in the ascorbate content may signify one of the mechanisms evolved by the plants for protection against reactive species of oxygen generated during photoinhibition particularly at low temperature (Nakano and Asada, 1987).

4.2.4.4 Ascorbate peroxidase

Ascorbate peroxidase is an important enzyme which scavenges \(\text{H}_2\text{O}_2\) in higher plant chloroplasts (Asada and Takahashi, 1987). Hydrogen peroxide is the first stable product of both monovalent and divalent reduction of oxygen. However, no
Fig. 4.7 Changes in ascorbate content in leaf extracts prepared from unstressed and photoinhibited leaves with PFD of 600 W.m\(^{-2}\) at 25\(^\circ\)C (open circles) and 8\(^\circ\)C (filled circles) for different periods of time. Results are expressed in folds of the initial ascorbate content. Each point represent mean of three separate experiments.
divalent reduction of oxygen has been demonstrated in normal chloroplasts and most of the hydrogen peroxide in chloroplasts has been suggested to be produced through the dismutation of superoxide catalyzed by SOD. An increase in ascorbate peroxidase activity was observed after photoinhibition of leaves with 600 W m\(^{-2}\) at both 25 and 8\(^\circ\)C. Ascorbate peroxidase activity increased by 1.3 folds on photoinhibition of leaves at 25\(^\circ\)C for 20h (Fig. 4.8a) whereas it increased to 1.8 folds on similar treatment at 8\(^\circ\)C (Fig. 4.8b). The increase in ascorbate peroxidase activity signifies an increase in H\(_2\)O\(_2\) production during photoinhibition of wheat leaves. The greater increase in activity during photoinhibition at 8\(^\circ\)C is consistent with the suggestions of increased rates of production of active oxygen species during photoinhibition at low temperatures. Under the conditions where the production rate of hydrogen peroxide is high, the biosynthesis of ascorbate peroxidase is reported to be increased (Gillham and Dodge, 1984; Tanaka et al., 1985). Thus it seems likely from our results that the increased production of hydrogen peroxide during photoinhibition led to the increased rate of biosynthesis of the enzyme.

Comparatively slower increase in ascorbate peroxidase activity was observed at both the temperatures on photoinhibition of leaves in which nuclear directed protein synthesis was inhibited (Fig. 4.8a and 4.8b). As in the cases
Fig. 4.8 Changes in ascorbate peroxidase activity in leaf extracts prepared from unstressed and photoinhibited leaves with PFD of 600 W.m\(^{-2}\) at 25°C (a) and 8°C (b) for different periods of time. Open circles: no addition, filled circles: 10 μg.ml\(^{-1}\) CHI, open triangles: 200 μg.ml\(^{-1}\) CAP. The seedlings were kept in CHI and CAP solutions for 12h prior to photoinhibition treatment. Results are expressed in folds of the initial level. Each point represent mean of three separate experiments.
of catalase and SOD, the chloroplast directed protein synthesis inhibitor CAP was inefficient in retarding the increase in activity on photoinhibition (Fig. 4.8a and 4.8b). These results indicate that the increase in activity of ascorbate peroxidase during photoinhibition was dependent on the biosynthesis of the enzyme which is likely to be nuclear encoded.

4.2.4.5 Monodehydroascorbate reductase

The monodehydroascorbate radicals are produced in the chloroplasts by several enzymatic reactions including ascorbate peroxidase and ascorbate oxidase, and by non-enzymatic reactions such as oxidation of ascorbate by superoxide and hydroxyl radicals (Nakano and Asada, 1981; Asada and Takahashi, 1987). The ascorbate peroxidase catalyzed reaction has been suggested to be the major source of monodehydroascorbate radical production in higher plant chloroplasts (Asada and Takahashi, 1987). Monodehydroascorbate reductase reduces the monodehydroascorbate radicals generated by ascorbate peroxidase and regenerates the ascorbate (Hossain et al., 1984) which is useful for the detoxification of active oxygen species (Hausladen and Kunert, 1990) and indirectly needed for the activities of several other enzymes such as ascorbate peroxidase (Nakano and Asada, 1987).
Monodehydroascorbate reductase activity in our experiments increased by 1.2 and 1.8 folds during photoinhibition of wheat leaves with 600 W.m\(^{-2}\) at 25 and 8\(^{\circ}\)C respectively (Fig. 4.9a and 4.9b). Monodehydroascorbate reductase is known to be regulated by its metabolites (Hossain et al., 1984; Wingate et al., 1988). Hence this increase in monodehydroascorbate reductase activity might result from an activation of the enzyme by increased production of monodehydroascorbate radicals.

The protein synthesis inhibitors CHI and CAP did not have any effect on the rate of increase in monodehydroascorbate reductase activity during photoinhibition of intact wheat leaves at both 25 and 8\(^{\circ}\)C (Fig. 4.9a and 4.9b). It indicates that this enzyme was regulated only by its substrate monodehydroascorbate radicals and the increase in activity during photoinhibition did not involve synthesis of any new enzyme.

4.2.4.6 Dehydroascorbate reductase

A gradual increase in dehydroascorbate reductase activity was observed on photoinhibition of wheat leaves at 25 and 8\(^{\circ}\)C (Fig. 4.10a and 4.10b). Unlike the other enzymes in the present study, its activity on photoinhibition of leaves at 8\(^{\circ}\)C was not significantly different than that at 25\(^{\circ}\)C. This
Fig. 4.9 Changes in monodehydroascorbate reductase activity in leaf extracts prepared from unstressed and photo-inhibited leaves with PFD of 600 W.m$^{-2}$ at 25°C (a) and 8°C (b) for different periods of time. Open circles: no addition, filled circles: 10 µg.ml$^{-1}$ CHI, open triangles: 200 µg.ml$^{-1}$ CAP. The seedlings were kept in CHI and CAP solutions for 12h prior to photoinhibition treatment. Results are expressed in folds of the initial level. Each point represent mean of three separate experiments.
could be because of the non-enzymatic nature of production of dehydroascorbate (Foyer and Halliwell, 1976; Schoner and Krause, 1990) which in turn regulates its activity (Nakano and Asada, 1981). Dehydroascorbate is produced in plants by disproportionation of monodehydroascorbate radicals (Law et al., 1983). Since the production of dehydroascorbate is non-enzymatic, it is unlikely to be affected by temperature and the activities of other enzymes involved in the reduction of ascorbate. The function of dehydroascorbate reductase has been suggested to feed back the molecules to the ascorbate pool that have escaped the monodehydroascorbate reductase reaction and converted to dehydroascorbate (Asada and Takahashi, 1987; Schoner and Krause, 1990). This is also evident from the fact that the protein synthesis inhibitors CHI and CAP also did not have any effect on the activity of this enzyme (Fig. 4.10a and 4.10b).

4.2.5 Lipid peroxidation

Peroxidation of lipids is defined as the oxidative deterioration of polyunsaturated lipids (Halliwell and Gutteridge, 1989). Approximately half of the chloroplast dry weight is composed of lipid and lipid soluble components, 20% of which being the photosynthetic pigments (Wise and Naylor, 1987). The presence of lipids with polyunsaturated fatty acyl residues is a characteristic feature of higher plant thylakoid
Fig. 4.10 Changes in dehydroascorbate reductase activity in leaf extracts prepared from unstressed and photo-inhibited leaves with PFD of 600 W.m$^{-2}$ at 25°C (a) and 8°C (b) for different periods of time. Open circles: no addition, filled circles: 10 μg.ml$^{-1}$ CHI, open triangles: 200 μg.ml$^{-1}$ CAP. The seedlings were kept in CHI and CAP solutions for 12h prior to photoinhibition treatment. Results are expressed in folds of the initial level. Each point represent mean of three separate experiments.
membranes (Leech and Murphy, 1976; Murphy, 1986). These unsaturated lipids of thylakoid membrane make the chloroplasts susceptible to peroxidative degradation.

Exposure of leaves to 600 W.m\(^{-2}\) for 10h at 25\(^{\circ}\)C caused a 2.3 fold increase in MDA production (Fig. 4.11a). Light treatment given in presence of CHI and CAP produced a 2.8 and 3.3 fold increase in the rate of lipid peroxidation respectively (Fig. 4.11a).

High light stress at low temperature stimulated the peroxidation of thylakoid lipids in wheat leaves. Exposure of leaves to 600 W.m\(^{-2}\) for 10h at 8\(^{\circ}\)C increased the rate of lipid peroxidation by approximately 2.7 folds of the initial level (Fig. 4.11b). The extent of thylakoid lipid peroxidation increased by 3.2 folds in the leaves photoinhibited with 600 W.m\(^{-2}\) for 10h at 8\(^{\circ}\)C in presence of CHI (Fig. 4.11b) whereas it increased by 2.8 folds after the same treatment at 25\(^{\circ}\)C (Fig. 4.11a). These results show a marked stimulation of lipid peroxidation on photoinhibition of leaves in presence of CAP. The same treatment to leaves at 8\(^{\circ}\)C caused a 3.9 fold increase in lipid peroxidation compared to a 3.3 fold increase at 25\(^{\circ}\)C (Fig. 4.11a and 4.11b).

These observations suggest involvement of active oxygen species which may be involved in the initiation and
**Fig. 4.11** Peroxidation of thylakoid lipids as a function of period of light treatment in absence and presence of protein synthesis inhibitors. Open circles: no addition, filled circles: 10 µg.ml⁻¹ CHI, open triangles: 200 µg.ml⁻¹ CAP. The seedlings were kept in CHI and CAP solutions for 12h prior to photoinhibition treatment. Results are expressed as percent of initial value. Wheat leaves were exposed to a photo-inhibitory light of 600 W.m⁻² at 25°C (panel a) and 8°C (Panel b). Each point represent mean of three separate experiments.
propagation of thylakoid lipid peroxidation. The higher extent of thylakoid lipid peroxidation during photoinhibition at \(8^\circ\text{C}\) is in agreement with the electron transport data and suggests an increase in the production of oxidative species during photoinhibition at low temperature.

Photoinhibition of photosynthesis is known to result in the photodegradation of the chloroplast encoded Dl protein. Despite the extensive work done on photoinhibition, its mechanism is not completely understood as yet. The increase in activities of all the antioxidant enzymes under investigation except for catalase and DHAR suggest an increase in the production of active oxygen species during photoinhibition particularly at low temperature. The increase in activities of these enzymes are suggested to be due to their biosynthesis which is evident from our results of photoinhibition in presence of CHI. All these enzymes are likely to be encoded by the nuclear genome as the extent of damage was more in the leaves photoinhibited in presence of CHI and CAP did not show a significant effect. The reduction in catalase during photoinhibition was due to photoinactivation of the enzyme at high light intensities which has been reported earlier (Feierabend and Engel, 1986). The activity of DHAR was also not altered on photoinhibition as it is known to be regulated by its substrate DHA which is produced
non-enzymatically in the chloroplasts. Our results therefore suggest an involvement of active oxygen species which might damage the photosynthetic apparatus and result in the reduction of photosynthetic electron transport and chlorophyll fluorescence characteristics. The generation of oxy-radicals during photoinhibition is also substantiated by the peroxidation of thylakoid lipids.