Inhibition of electron transport at the cytochrome b_6f complex protects photosystem II from photoinhibition

A. Krieger-Liszkay[a], K. Kienzler[a], G.N. Johnson[b,*]

[a] *Institute of Biology II, University of Freiburg, Schänzlestr. 1, 79104 Freiburg, Germany*
[b] *School of Biological Sciences, University of Manchester, 3.614 Stopford Building, Oxford Road, Manchester M13 9PT, UK*

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Abstract Photoinhibition of photosystem II (PS II) activity was studied in thylakoid membranes illuminated in the presence of the inhibitor of the cytochrome b_6f complex 2′iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenylether (DNP-INT). DNP-INT was found to decrease photoinhibition. In the absence of DNP-INT, anaerobosis, superoxide dismutase and catalase protected against photoinhibition. No effect of these treatments was observed in the presence of DNP-INT. These data demonstrate that photoinhibition under these conditions is caused by reactive oxygen species which are formed most probably by the reduction of oxygen at photosystem I. The results are discussed in terms of the importance of photosynthetic control in protection against photoinhibition in vivo.

Key words: Photoinhibition; 2′Iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenylether; Reactive oxygen species

1. Introduction

When plants are exposed to an irradiance greater than can be used in photosynthesis, they are liable to be damaged in a process called photoinhibition. In recent years, a large number of studies have shown that the primary site of damage in high light is the photosystem II (PS II) reaction centre (for a review see [1]). A number of different mechanisms for this damage have been identified, that invoke damage to the donor side or the acceptor side of the reaction centre. Donor-side photoinhibition is thought to occur under conditions where the donation of electrons to the primary donor, P680, is impaired, due to damage to the oxygen evolving complex. In the light, highly oxidising species such as P^+_{680} and/or Tyr^+_Z can accumulate which promote photoinhibition. Acceptor-side inhibition is thought to occur when the flow of electrons away from PS II is limited. Under these conditions, a long-lived chlorophyll triplet state becomes detectable [2] which can react with oxygen to form $^1O_2$, the reactive oxygen species (ROS) which is thought to be responsible for photodamage [3,4].

Although PS II is held to be the most vulnerable component of the photosynthetic apparatus, other complexes are liable to damage under some circumstances. The reaction centre of photosystem I can be damaged by high light, most notably when plants are exposed to extreme temperatures [5]. The mechanism of this damage is not well understood but may involve the generation of oxygen radicals at or near the acceptor side of PS I. Iron–sulphur centres bound to PS I or ferredoxin are known to be able to reduce molecular oxygen to form superoxide; the Mehler reaction [6]. Dismutation of superoxide, spontaneously or catalysed by superoxide dismutase (SOD), generates hydrogen peroxide. In the presence of reduced transition metal ions hydrogen peroxide can form hydroxyl radicals; the Fenton reaction. These and other radical species can cause widespread damage to the cell, including damage to proteins and peroxidation of lipids. The above reactions are likely to be involved in the bleaching of photosynthetic tissues seen in extreme high light.

It has long been known that the presence of a high pH gradient (ΔpH) across the thylakoid membrane inhibits electron flow through the thylakoid electron transport chain; 'photosynthetic control'. This inhibition is thought to be due to the effect of a high proton concentration on the deprotonation and oxidation of plastoquinol at the lumenal face of the cytochrome b_6f complex. It has been suggested that, under conditions where demand for reductant in carbon fixation is limited, for example by insufficient CO_2 supply, photosynthetic control might act to limit the extent of the Mehler reaction, so limiting the production of radical species [7,8]. Alternatively, it has been suggested that the Mehler reaction may actually be beneficial, preventing over-reduction of the electron transport chain, so protecting PS II from acceptor-side photoinhibition [9].

Here, we show that inhibiting the flow of electrons from PS II to PS I, thus inhibiting the Mehler reaction, has the effect of protecting PS II from photoinhibition. We demonstrate that this effect is related to the presence of oxygen, implicating ROS in the damage. This view is supported by the observation that enzymes of the antioxidation system decrease the extent of photoinhibition. The implications of these observations for regulation of photosynthesis are discussed.

2. Materials and methods

Intact chloroplasts were isolated from spinach, grown in a controlled growth room, using the method described by Laasch [10]. Prior to each measurement, chloroplasts were osmotically shocked in a solution containing 7 mM $MgCl_2$ and 15 mM HEPES (pH 7.6) for 15 s. After this time an equivalent volume of a solution containing 0.6 M sorbitol, 7 mM $MgCl_2$ and 15 mM HEPES (pH 7.6) was added to increase the osmotic potential. For all photoinhibition experiments, shocked chloroplasts were illuminated with white light of 2200 μmol m^{-2} s^{-1}. Photoinhibition treatments were conducted in the presence of reagents as indicated in the text and figure legends. Following treatment, photosynthetic capacity was estimated as the rate of oxy-

*Corresponding author. Fax: (44)-161-275 3938.
E-mail: giles.johnson@man.ac.uk

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gen evolution recorded at a saturating light intensity (5000 μmol m^{-2} s^{-1}) in the presence of 10 mM NH_4Cl, 500 μM 2,6-*p*-phenylbenzochinone (pPBQ) and 1 mM potassium ferricyanide. Oxygen evolution was recorded using a Hansatech oxygen electrode fitted to a DW2 liquid phase electrode chamber (Hansatech, King's Lynn, UK). Photoinhibitory treatments were performed in the same chamber. Light was supplied to the chamber from a Schott KL1500 lamp (Schott, Germany), passed through a glass fibre optic bundle. All measurements were performed at a temperature of 20°C.

3. Results

2′Iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenylether (DNP-INT) is an inhibitor of the cytochrome b_6f complex that binds to the lumenal (Q_o) plastoquinol-oxidising quinone binding site, thereby inhibiting plastoquinol oxidation [11]. When thylakoid membranes were subjected to photoinhibitory illumination in the presence of DNP-INT (but in the absence of electron acceptors other than oxygen) the extent of inhibition of PS II activity was substantially reduced (Fig. 1). This effect was most pronounced in the first 5 min of photoinhibitory illumination. At the concentration used (5 μM) DNP-INT has only a marginal effect on PS II electron transport (see legend to Fig. 1) whereas the reduction of ferricyanide by PS I is inhibited by more than 90%, as shown previously [11]. PS II activity in the presence of DNP-INT was additionally assayed by thermoluminescence. In the presence of DNP-INT a normal B-band at 35°C was observed which originates from a $S_2Q_B^-$ recombination (data not shown).

The presence of uncouplers (NH_4Cl or nigericin) stimulated the extent of photoinhibition as has been seen previously [12]. In the presence of DNP-INT no such effect of uncouplers was observed (Fig. 1).

In order to test whether the damage seen in the above experiment is due to the formation of oxygen radicals, we performed photoinhibition experiments in the absence of oxygen. Removal of oxygen partially protects PS II from light-induced damage, suggesting that the damage seen is at least partially caused by reactions involving molecular oxygen (Fig. 2). When DNP-INT was added under anaerobic conditions, the same extent of loss of PS II activity was observed as in the presence of oxygen. Thus, we conclude that the protective effect of DNP-INT is due to its ability to inhibit the formation of ROS.

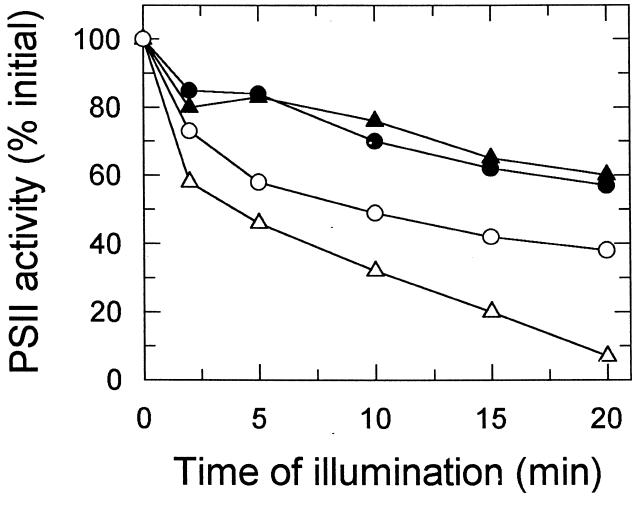

Fig. 1. Effect of DNP-INT and uncoupler on photoinhibition of PS II activity of spinach thylakoid membranes. Open symbols: no addition, closed symbols: addition of 5 μM DNP-INT, circles: no uncoupler, triangles: addition of 5 mM NH_4Cl during the photoinhibitory illumination. The samples were illuminated with white light (2200 μmol quanta m^{-2} s^{-1}) at 20°C for the time indicated. Oxygen evolution was measured using 0.5 mM pPBQ as electron acceptor and 10 mM NH_4Cl as uncoupler. The activities prior to photoinhibition were the following: no addition: 384 μmol O_2 mg chl^{-1} h^{-1}, in the presence of 5 μM DNP-INT: 354 μmol O_2 mg chl^{-1} h^{-1}.

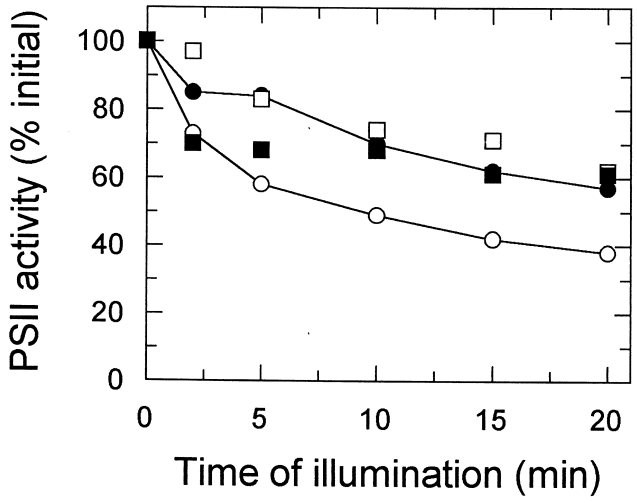

Fig. 2. Photoinhibition of thylakoid membranes under anaerobic (squares) and aerobic (circles) conditions. open symbols: no addition, closed symbols: addition of 5 μM DNP-INT. Anaerobiosis was obtained by flushing the buffer with argon prior to the addition of the thylakoid membranes and during the photoinhibitory illumination. The photoinhibition and the activity measurements were performed as in Fig. 1. Activities: no addition: 340 μmol O_2 mg chl^{-1} h^{-1}, in the presence of 5 μM DNP-INT: 320 μmol O_2 mg chl^{-1} h^{-1}.

Oxygen radicals can be formed at PS I through the Mehler reaction, generating superoxide which is transferred by SOD to hydrogen peroxide. In the presence of transition metals, hydroxyl radicals can be produced from hydrogen peroxide. Additionally, charge recombination reactions in the photosystems can lead to the formation of triplet excited chlorophyll which can in turn act as a sensitiser for the formation of singlet excited oxygen. In order to test whether the formation of superoxide and/or hydrogen peroxide is involved in the inhibition of PS II observed in our experiment, rather than singlet oxygen, we examined the effect of addition of SOD and catalase on the kinetics of photoinhibition (Fig. 3A). Addition of SOD decreased the rate of photoinhibition. Catalase had even a bigger protective effect, the magnitude of which was similar to that observed upon addition of DNP-INT. No synergistic effects were observed upon addition of both catalase and SOD. Addition of BSA did not affect the rate of photoinhibition, indicating that the effects observed were related to the catalytic activity of the enzymes, rather than to a non-specific stabilising effects of the protein. Addition of azide, a quencher of singlet oxygen, did not protect PS II from photoinhibition, either in the presence or absence of DNP-INT (data not shown).

When the same additives were tested in the presence of DNP-INT, practically no protection effects were seen (Fig. 3B), indicating that the blockage of the linear electron flow

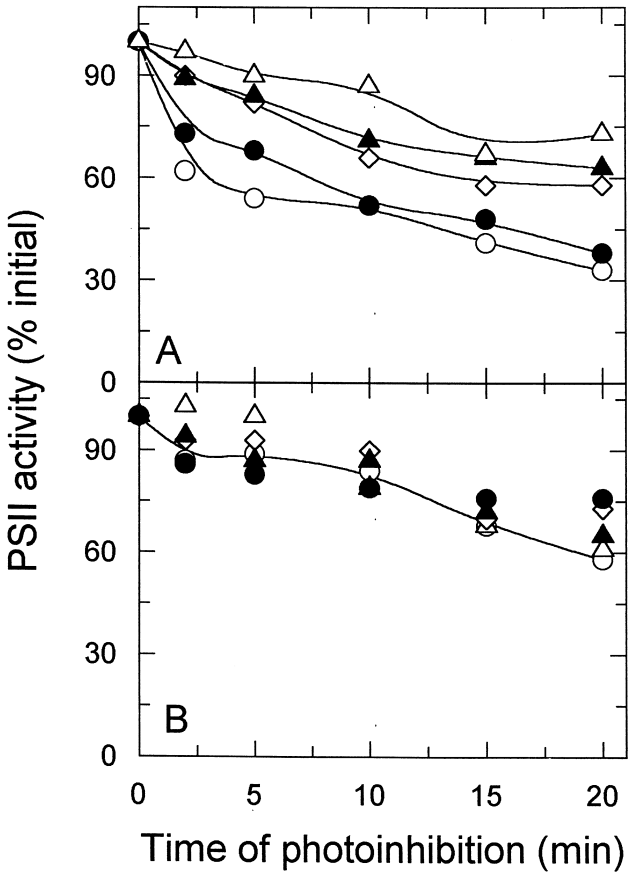

Fig. 3. Effect of antioxidative enzymes and BSA on photoinhibition of thylakoid membranes in the presence (A) and absence (B) of 5 μM DNP-INT. Open symbols: no addition of antioxidative enzymes or BSA, filled symbols: addition of antioxidative enzymes or BSA: squares: SOD; diamonds: catalase; triangles: catalase+SOD; circles: BSA. The following concentrations were used; SOD: 255 units ml^{-1}; catalase: 1250 units ml^{-1}; BSA: 0.2 mg ml^{-1}.

through the cytochrome b_6f complex and PS I prevented the formation of ROS.

In order to show that the effect of DNP-INT was not related to a direct effect on PS II or to any ability this reagent has to scavenge oxygen radicals, the effects of DNP-INT on isolated PS II membranes ('BBY's') was tested. DNP-INT did not protect such preparations from photoinhibition (data not shown).

PS I is found to be much less sensitive to photoinhibition than is PS II. Under the conditions used in the experiments shown here, no damage was observed to PS I, as measured by the rate of oxygen uptake in the presence of methyl viologen, ascorbate, 2,6-dichlorophenol-indophenol and DCMU (data not shown).

4. Discussion

Data presented in this paper demonstrate clearly that inhibition of the thylakoid electron transport chain at the level of the cytochrome b_6f complex protects PS II against photoinhibition (Fig. 1). The absence of this effect in the absence of oxygen indicates that the protection observed is related to an inhibition of reactive oxygen formation (Fig. 2). This view is further supported by the observation that enzymes involved in the scavenging of ROS protect in the absence but not the presence of DNP-INT (Fig. 3).

DNP-INT might protect PS II from active oxygen either by preventing ROS formation in the PS II reaction centre itself or by preventing the generation of radical species elsewhere that then diffuse to and damage PS II.

Protection against photoinhibition by inhibitors of the PS II acceptor side has previously been reported [13–16]. DCMU protects against light induced damage of PS II activity [15,16] and retards the turnover of the D1 protein [13,14,16] however these effects have usually been attributed to direct effects within the PS II reaction centre. DCMU binds to the Q_B-binding site of PS II and inhibits the electron transport chain prior to the site of action of DNP-INT. It has been shown recently that the midpoint potential of the redox couple Q_A/Q_A^- is shifted in the presence of DCMU by approximately 50 mV to a more positive value than in the absence of the herbicide [17]. It was suggested that this change in the midpoint potential modulated the pathway of charge recombination reactions within PS II, reducing thereby the probability of chlorophyll triplet and consecutive $^1O_2$ formation. Thermoluminescence measurements indicate that, in the presence of DNP-INT, no such shift of the redox potentials of the quinones (Q_A or Q_B) at the acceptor side of PS II occurs. Indeed, there is no evidence that DNP-INT binds significantly to the PS II acceptor side at all. Nor does DNP-INT protect PS II from photoinhibition in BBY-type PS II membranes. Therefore, DNP-INT must protect PS II by a different mechanism to DCMU, although it cannot be excluded that DCMU provides additional protection in the same way as DNP-INT.

If DNP-INT is not affecting the PS II reaction centre directly, it must operate by preventing the formation of active oxygen species elsewhere. There is evidence from a number of sources that PS II can be damaged by free radicals produced outside the reaction centre. Chung and Jung [18] suggested that Fe–S centres in PS I and the cytochrome b_6f complex may give rise to $^1O_2$. The mechanism of this is unclear, however, it is not clear that this generation would be affected by DNP-INT. Furthermore, the absence of any protective effect of sodium azide, a quencher of $^1O_2$, tends to rule out direct involvement of this species under our conditions.

If damage to PS II is not due to $^1O_2$, other oxygen radicals must be involved. PS II has been shown to be sensitive to exogenous H_2O_2 [19]. Krieger-Liszkay and Rutherford [20] showed that Cl^--depleted PS II enriched membrane, that produce H_2O_2 upon illumination, can damage active PS II when a mixture of centres of both type are illuminated together. In the presence of a complete thylakoid electron transport chain, the Mehler reaction will give rise to the formation of superoxide and H_2O_2. DNP-INT, an inhibitor of linear electron transport, will inhibit the production of these, so protecting PS II from damage.

In the absence of uncouplers, electron transport to O_2 will generate a ΔpH which will inhibit electron transport (photosynthetic control), thus making the Mehler reaction self-limiting. Addition of an uncoupler removes that limitation, increases the rate of superoxide production and increases damage to PS II. Previous studies have suggested that increased photoinhibition in the presence of uncouplers is due to the inhibition of high energy-state quenching (qE) [12]. In the presence of DNP-INT, no ΔpH is generated and so no qE

is formed, thus the difference in photoinhibition seen here clearly cannot be due to qE.

A protective effect of inhibiting electron transport at the level of the b_6f complex has been observed in vivo in both algae and plants [21,22]. Zer et al. [21] suggested that this effect may be due to an inhibition of the turnover of the PS II reaction centre. Our results point to an alternative interpretation – that it is electron transport though PS I and the resultant production of oxygen radicals that damages PS II. Although there is evidence for regulation of the electron transport chain in vivo [7,8], it has not been well studied. Such regulation may play a vital role in protecting both PS II and the chloroplast in general from the damaging effects of ROS under conditions of environmental stress.

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