Mechanistic aspects of the inhibition of photosynthesis by light

Mechanistische aspekten van de remming van de fotosynthese door licht

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Mechanistic aspects of the inhibition of photosynthesis by light

Proefschrift
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in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen
in het openbaar te verdedigen
op vrijdag 5 januari 1996
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen

Stellingen

1   De vaststelling dat elektronentransport naar een elektronenacceptor plaats vindt in de aanwezigheid van een remmer is onvoldoende bewijs voor de conclusie dat dit elektrontransport systeem ongevoelig is voor die remmer.

    Dit proefschrift

2   Omdat er onvoldoende experimentele middelen beschikbaar zijn om de functie van bicarbonaat in fotosysteem II te bestuderen krijgt de regulerende rol van dit molekuul in dit fotosysteem onvoldoende aandacht.

    Dit proefschrift

3   2,5-Dimethyl-p-benzochinon accepteert in intacte cellen van cyanobacteriën niet specifiek elektronen van fotosysteem II.

    Dit proefschrift

4   De resultaten over de zuurstofproduktie capaciteit van fotosysteem II in bladeren verkregen met de toepassing van fotoakoustische spectroscopie zijn vergelijkbaar met fluorescentie data. Dit in tegenstelling tot resultaten verkregen met de zuurstof elektrode.

    Dit proefschrift

5   De Conclusie van Öquist et al. (1992), dat fotoinhibitie optreedt als meer dan 40% van de reactiecentra zich in de gesloten toestand bevindt, berust op een verkeerde interpretatie van de data.

    Öquist et al. (1992) Planta 186: 450-460

6   Bij de bestudering van de resistentiemechanismen van Penicillium italicum tegen ergosterol biosynthese remmers wordt te weinig aandacht besteed aan de rol van sterol-transporterende eiwitten.

7   Vergelijking van *Agrobacterium tumefaciens* en het wortelknobbelaaltje, wat betreft de fysiologie van hun plant-pathogeen interactie en hun brede waardplant reeks, suggereert dat het wortelknobbelaaltje op een manier vergelijkbaar aan die van *Agrobacterium tumefaciens* ingrijpt in de hormoonhuishouding van zijn gastheer.

8   De wens van de overheid om over de oplossingen van de problemen van vandaag terstond te beschikken zou de financiële ondersteuning van fundamenteel onderzoek sterk moeten stimuleren.

9   Het idee van Macchiavelli dat staatsvormen door degeneratie cyclisch in elkaar overgaan benadert de werkelijkheid waarschijnlijk beter dan de postulatie van een ideale staatsvorm.

     Macchiavelli N, The Discourses

10   De overgang van het nomadische verzamelaars en jagers bestaan naar de sedentaire landbouw is één van de meest ongelukkige ontwikkelingen uit de geschiedenis van de mensheid.

11   De verkoop van Alaska door de Russische tsaar aan de Amerikanen was een verstandige beslissing.

     Bobrick B, Siberië: het land achter de horizon

12   Het feit dat de Eerste Wereldoorlog twee jaar langer kon duren door de uitvinding van een procédé voor het maken van stikstofverbindingen langs industriële weg illustreert de invloed van de wetenschap op de loop van de geschiedenis.

     Molenaar L, Chemie en Samenleving

13   Om het autogebruik in Nederland terug te dringen zou een verveelvoudiging van de wegenbelasting op een tweede auto binnen één huishouden een effectieve maatregel zijn.

Stellingen bij het proefschrift "Mechanistic aspects of the inhibition of photosynthesis by light".

Wageningen, 5 januari 1996.   Gert Schansker

... het verleden rot in de toekomst
een verschrikkelijk carnaval van dode bladeren

Anna Akhmatova
Epos zonder held

Voorwoord

Toen ik 5 jaar geleden als planteziektenkundige met mijn promotie onderzoek begon was mij niet geheel duidelijk waarom zoveel tijd en energie wordt gestoken in fotosynthese onderzoek. Nu, 5 jaar later, zie ik verschillende toepassingen voor de kennis vergaard over fotosynthese in bijvoorbeeld diagnostiek en modellering van planten. Ik geloof echter nog steeds niet in het aanwenden van deze kennis voor het verbeteren het fotosynthese proces noch voor het maken van artificiële systemen voor de omzetting van licht in chemische energie die ook maar in de verste verte op het origineel lijken. Desalniettemin heb ik gedurende de afgelopen jaren met plezier aan mijn onderzoek gewerkt. Het onderwerp, fotoinhibitie, heeft me van het begin af aan aangesproken. Het ontrafelen van het mechanisme van dit proces was een uitdaging. De beschikbaarheid van grote hoeveelheden elkaar vaak schijnbaar tegensprekende data maakten het tot een leuke puzzel. Ik heb geen overtuigend bewijs gevonden voor de ontwikkelde hypothese, maar een troost is, dat de vele anderen die aan het onderwerp werken of gewerkt hebben in dit opzicht ook gefaald hebben tot nu toe.

De laatste 5 jaar heeft de nodige veranderingen gebracht. De vakgroep Planten-fysiologisch Onderzoek waar ik begonnen ben bestaat niet meer en ook de werkomgeving is veranderd door een fusie en een verhuizing naar de banaan. De sfeer heeft er niet onder geleden, die was steeds goed al ging de inkrimping vorig jaar niet onopgemerkt voorbij.

Voor een promotie zijn promotoren nodig. Jacques, je hebt je een kundig manager van mijn promotie onderzoek getoond. Je bent altijd makkelijk toegankelijk geweest, je hebt me gestimuleerd om naar internationale congressen te gaan en om een poging te doen een Fulbright Scholarship te bemachtigen. Daarvoor mijn dank. Je hebt me echter er niet van kunnen overtuigen dat een ad hoc benadering van wetenschap de beste methode is. Ook Wim Vredenberg wil ik bedanken voor alle energie die hij gestoken heeft in de bespreking van het proefschrift. Ik ben geen fysicus en kijk soms op een andere niet mathematische wijze tegen een onderwerp aan. Dit heeft soms geleid tot misverstanden en lange discussies, maar ik denk dat de discussies het proefschrift ten goede zijn gekomen.
Van het groepje AIO's/OIO's dat in 1990/1991 begonnen is mag ik met mijn promotie de spits afbijten. Corine, Victor en Tijmen, ik wens jullie veel sterkte met het afronden van jullie proefschriften.

Verder wil ik Iris Houba en Oscar de Vos bedanken voor de voor mij verrichte experimenten. Iris, ik kan alleen maar hopen dat het geven van onderwijs een socialere

bezigheid is, dan het doen van onderzoek. Leen wil ik bedanken voor de goede kwaliteit erwten die hij voor mij kweekte.

Het eens in de week spelen van tennis tijdens de middagpauzes heeft ongetwijfeld een positieve invloed gehad op het uitvoeren van mijn onderzoek. Hans, bedankt, dat je gedurende de afgelopen jaren daarbij mijn sparring partner wilde zijn.

De Britse marine parafraserend zou je over wetenschap kunnen zeggen: 'Join science en see the world'. Ik ben in de afgelopen jaren in de gelegenheid gesteld om heel wat van de wereld te zien en heel wat mensen te ontmoeten. Mijn promotor Wim Vredenberg heeft wel een beetje gelijk als hij zegt, dat het inkomen van een AIO/OIO misschien niet geweldig is, maar dat de secundaire arbeidsvoorwaarden goed zijn. Mijn buitenlandse reizen vallen in drie delen uiteen: congresbezoek, het verblijf in de Verenig-de Staten en het begeleiden van de T33-excursie naar Hongarije. Wat betreft mijn verblijf in het lab van Wim Vermaas in Tempe (Arizona) wil ik de CIES (Council for Internatio-nal Exchange of Scholars) bedanken voor het mij toekennen van een Fulbright scholars-hip. Wim Vermaas wil ik bedanken voor de geboden gastvrijheid en de discussies. Op een gegeven moment heb ik me wel afgevraagd of je suggestie voor de meest geschikte mutant voor het onderzoek een goede was, maar het ziet er nu toch naar uit dat het een juiste keuze was. Further, I would like to thank Sveta, Cathy, Amethyst and Hadar. Without their presence, my stay would not have been such a pleasant one and I would never have seen the vermillion flycatcher. I also have to mention Pradip. Our discussions were as long as fruitless, but they were memorable.

Verder bewaar ik goede herinneringen aan de excursie naar Hongarije. De organisatie aan Hongaarse kant was heel goed en de samenwerking met de andere begeleiders liep ook naar wens. En, Chantal, ik kan alleen maar hopen, dat je nog eens de gelegenheid wordt gesteld de perfecte excursie te organiseren.

Van de verschillende bijeenkomsten die ik heb bijgewoond is vooral de NATO summer-school in Volterra goed bevallen. De formule van deze summerschools is uitermate geschikt voor het opdoen van wetenschappelijke kennis maar ook voor het ontmoeten van mensen.

Naast de buitenlanders die ik in het buitenland ben tegen gekomen waren er ook een aantal op het lab die mijn weg gekruist hebben. Manoj, I have good memories of your stay in the lab and the discussions we had. Professor Govindjee I would like to think for the interest he has shown in my research during his visits to Wageningen. The last foreigner I would like to mention is Vika. Although our paths crossed only for a few months I will remember the fox, the hedgehog and the sea for quite a while.

Contents

List of abbreviations

ATPase	thylakoid membrane H^+-ATP synthetase complex
atrazine	2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine
B-band	thermoluminescence band associated with $S_2Q_B^-$ charge recombination
C-band	thermoluminescence band peaking around 40-50 °C
CPx	chlorophyll binding proteins with a molecular weight x
cyt b_{559}	cytochrome b_{559}; integral part of Photosystem II
cyt b_6/f	cytochrome b_6/f complex, consists of two cytochromes b_{563}, a cytochrome f and a Rieske iron protein
D1 protein	Photosystem II reaction center protein containing the Q_B-binding site
D2 protein	Photosystem II reaction center protein to which Q_A is bound
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCBQ	2,6-dichloro-p-benzoquinone
DCPIP	dichlorophenolindophenol
dinoseb	2,4-dinitro-6-s-butylphenol
DMBQ	2,5-dimethyl-p-benzoquinone
DNOC	dinitro-o-cresol
ε(400nm)	extinction coefficient for silicomolybdate at a wavelength of 400 nm
F_m	fluorescence level when all Photosystem II reaction centers are closed
F_o	fluorescence level when all Photosystem II reaction centers are open
F_v	variable fluorescence; F_m-F_o
F_o'	minimum fluorescence level reached after turning off the actinic light
F_m'	yield of chl a fluorescence due to a saturating light pulse under steady state conditions
F	steady state fluorescence level in the light
F_v/F_m	efficiency of Photosystem II in dark adapted leaves
FeCy	potassium ferrihexacyanide
FNR	ferredoxin-$NADP^+$ oxidoreductase
ioxynil	3,5-diiodo-4-hydroxy-benzonitrile
I_{50}-value	concentration of a compound inhibiting 50% of electron transport activity
J	electron flux through Photosystem II under steady state conditions
K_b	concentration of a compound giving 50% binding to a specific binding site
K_m	substrate concentration giving half maximal electron transport activity
KCN	potassiumcyanide

LHCII	light harvesting complex protein of Photosystem II
MV	methylviologen; 1,1-dimethyl-4,4'-bipyridylium-dichloride
OD_{730}	optical density at 730 nm; measure for the cell density of a *Synechocystis* culture
P_{680}	reaction center chlorophyll of Photosystem II
P_{700}	reaction center chlorophyll of Photosystem I
PC	plastocyanine
pfd	photon flux density
pLHCII	precursor protein of light harvesting complex II
PpBQ	phenylparabenzoquinone
PQ	native plastoquinone molecule of Photosystem II
PQH_2	plastoquinol
PSIIα	Photosystem II reaction center localized in the grana region, active in linear electron transport
PSIIβ	Photosystem II reaction center, localized in the stroma lamellae, inactive in linear electron transport, has a smaller antenna system than PSIIα centers and is less sensitive to photoinhibition than PSIIα centers
Q_A	primary quinone electron acceptor of Photosystem II
Q_B	secondary quinone electron acceptor of Photosystem II
Q-band	thermoluminescence band associated with $S_2Q_A^-$ recombination
q_E	energy dependent quenching
q_I	photoinhibition dependent quenching
q_N	non-photochemical quenching
q_P	photochemical quenching
q_T	state-transition dependent quenching
RC	reaction center of Photosystem II
S_2	oxidation state of the water-splitting system
SiMo	silicomolybdate
SiTu	silicotungstate
SOD	superoxide dismutase
V_{max}	maximum electron transport activity
ϕ_P	quantum yield of Photosystem II electron transport

1 General Introduction

Photosynthesis: adaptation and regulation
A plant is an immobile organism. It has a fixed standing place and cannot escape from stress conditions. This confronts the plant with a dilemma. A plant depends on light energy for growth and development. To function well under low light conditions it is built to absorb as much light as possible: the area of plant tissue exposed to light is high and in the chloroplasts the membranes are extremely folded to further extend the surface for light absorption. Under high light conditions the plant is faced with an excess of light energy that has to be dissipated to prevent damage. Plants have developed several mechanisms of adaptation and regulation to function efficiently under both high and low light conditions.

Mechanical adaptation mechanisms
Several plant species are able to change the leaf orientation relative to the sun for altering the effective absorption cross section of the leaf: under low light optimizing their absorption cross section and under high light minimizing it (Koller 1986). In a cell, chloroplasts can achieve the same goal by aligning themselves differently in the cell. Under low light conditions the chloroplasts will move to the cell membrane at the top or bottom side of the cell, optimizing their absorption cross section. Under high light conditions the chloroplast will move to that part of the cell membrane perpendicular to the leaf surface. The effect is self shading, which minimizes the absorbance cross section (Haupt and Scheuerlein 1990).

Adaptation of plants to the growth irradiance at the cellular level
Within the leaf a long term adaptation to the ambient irradiance occurs. There exists a steep light gradient within a leaf (Terashima and Saeki 1983) and this gradient influences the photosynthetical characteristics of chloroplasts. Terashima and Inoue (1985) and Terashima *et al.* (1986) showed in a very elegant way the heterogeneity and flexibility of chloroplasts within a leaf. For leaves of spinach and *Glycine max* two observations were made: 1. The light gradient determines the characteristics of a chloroplast within a leaf creating a gradient of photosynthetic properties (Terashima and Inoue 1985) and 2. In

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developing leaves this gradient of photosynthetic properties can be inverted by turning the leaf around (Terashima *et al.* 1986). This means that, at least in C3 plants, the photosynthetic characteristics of chloroplasts within the leaf are not a characteristic of a particular cell type but depend solely on the light environment of that chloroplast.
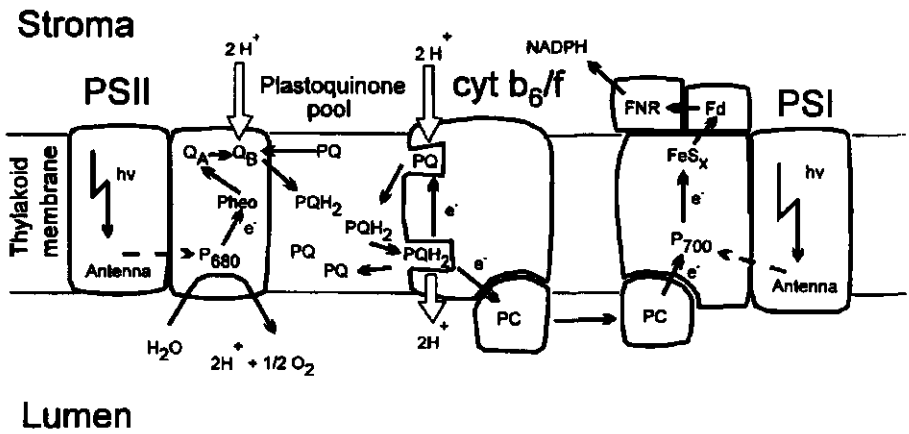

Fig. 1.1. Scheme of electron and proton fluxes through the photosynthetic electron transport chain. The scheme as it is drawn supposes a double turnover of PSII and PSI to account for the two electrons required for the redox reaction of PQ/PQH_2. For a discussion of the reactions see Chapter 2. P_{680}: reaction center chlorophyll of PSII; Pheo: pheophytin; Q_A: primary quinone electron acceptor; Q_B: secondary quinone electron acceptor; PQ: plastquinone; PQH_2: plastoquinol; PC: plastocyanin; P_{700}: reaction center chlorophyll of PSI; FeS_x: one of the iron sulfur clusters; Fd: ferredoxin; FNR: ferredoxin-NADPH-reductase.

Adaptation of the electron transport chain
Thylakoid membranes contain the four protein complexes that are required to convert ADP to ATP and NADP$^+$ to NADPH with light as energy source: Photosystem II, cytochrome b_6/f-complex, Photosystem I and ATPase. The first three complexes create the reductive power necessary to reduce NADP$^+$ and pump protons into the lumen, and the fourth one, ATPase, uses the proton gradient to phosphorylate ADP. In Fig. 1.1 electron and proton transport in the thylakoid membrane are illustrated.

In mature leaves many regulatory processes are mediated by the balance between the photon flux density and metabolic processes as a source and a sink, respectively. The chloroplast is able to sense the redox state of certain components of the electron transport chain and to adjust imbalances between supply and demand (see Bennett 1991, Allen 1992

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for reviews). Another mechanism by which the electron transport chain is regulated is feedback regulation caused by the low pH in the lumen created during electron transport and coupled proton import into the lumen (Dau 1994).

An imbalance between the formation and the consumption of NADPH will lead to a change in the redox state of the cytochrome b_6/f-complex. If the consumption of NADPH is lower than the formation of NADPH this complex will get more reduced, activating a kinase which phosphorylates LHCII (Coughlan and Hind 1986a, b), a part of the antenna system of PSII. Phosphorylated LHCII detaches itself from PSII making the absorption cross section of PSII smaller (Horton and Black 1982, Steinback *et al.* 1982, Horton and Lee 1984). In the opposite case the cytochrome b_6/f-complex becomes more oxidized, inactivating the kinase and allowing a phosphatase to dephosphorylate LHCII (Bennett 1984). Subsequently this antenna complex can attach itself again to PSII.

Electron transport is also regulated by the pH in the lumen. Both water splitting and the reoxidation of plastoquinol deliver two protons to the lumen. These protons are used to drive ATP synthesis. A change in the equilibrium between lumen acidification and ATP synthesis will change the pH of the lumen. The lumen pH regulates photosynthesis both on the fast and medium time scale. A low lumen pH slows down the reoxidation of plastoquinol (Hope *et al.* 1994). As the reoxidation of plastoquinol is the slowest step in the electron transport chain (Stiehl and Witt 1969, Witt 1971) this attenuates the electron transport rate. This is called photosynthetic control (West and Wiskich 1968). Another process that is induced by a low lumen pH is the formation of zeaxanthin from violaxanthin (Hager 1969). Formation of zeaxanthin affects the structure of the antenna of PSII increasing the probability that a photon is quenched in the antenna system (Horton and Ruban 1992) and thus lowering the source strength of PSII. In Fig. 1.2 these processes are illustrated.

On a timescale of days changes in the photon flux density will also induce adaptation of the stoichiometry of components of the photosynthetic apparatus to optimize the use of light both on the plant level as well as within a leaf (Wild *et al.* 1973, Wild *et al.* 1986, Terashima and Inoue 1985, Chow and Anderson 1987a,b). These long term adaptations are also induced by changes in the redox state of the electron transport chain (Melis 1992, Anderson *et al.* 1992). It is unclear which component of the electron transport chain signals the synthetic machinery to change its pattern of synthesis. The cytochrome b_6/f-complex would be a good candidate as it is the only complex for which a good correlation between growth irradiance, electron flow and the content of cyt b_6/f was found (Evans 1987).

The process of photoinhibition, which will be discussed below, is a special case as it is a forced form of adaptation.

The D1 protein of Photosystem II and photoinhibition
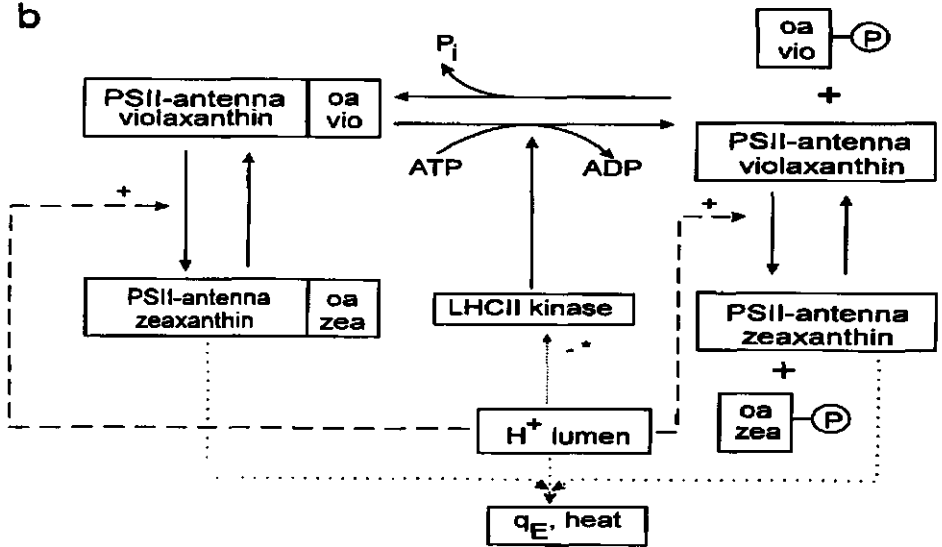The crystallization of the reaction center of purple bacteria (Deisenhofer *et al.* 1984,

a

b

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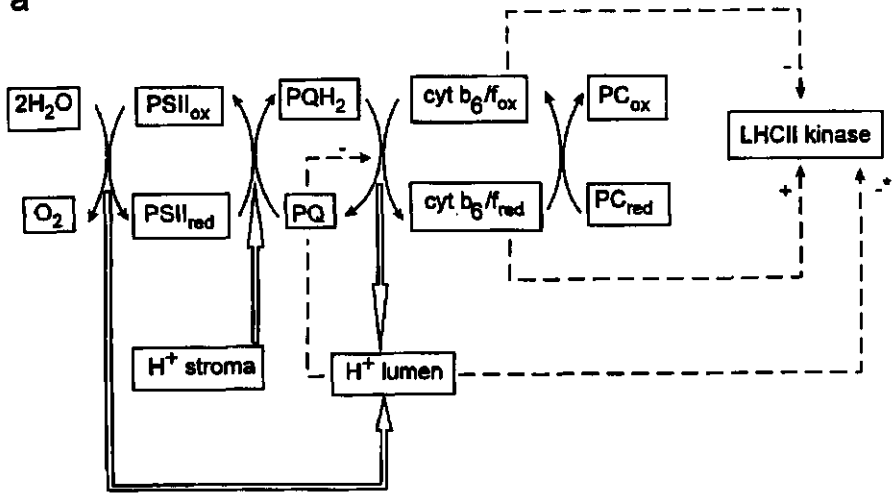
Fig. 1.2. Two schemes illustrating the regulation of electron transport by the redox state of the cytochrome b₆/f-complex and the lumen pH. a. Electron transport chain: The solid arrows indicate electron transport and open arrows indicate the release or the uptake of protons. The broken lines indicate a stimulatory (+) or an inhibitory (-) effect on the activity of an enzyme or enzyme complex. A low lumen pH inhibits the activity of the LHCII kinase, indicated as -*. However, it is unknown whether there is a direct inhibitory effect on the kinase or that the low lumen pH stimulates zeaxanthin formation in LHCII (the substrate of the kinase) which might affect the binding affinity of the substrate for the kinase. b. PSII antenna system: The interaction between lumen pH, LHCII kinase, the antenna of PSII and the induction of energy quenching and heat release. The abbreviation oa stands for outer antenna, which means here the part of LHCII that can be phosphory- lated by LHCII kinase and disconnect from PSII. The other abbreviations are: P: phosphate group, vio: violaxanthin and zea: zeaxanthin. +, - and -* have the same meaning as in Fig. 1.2a.

1985) gave a new perspective to the structure of PSII. On the basis of homology between the reaction center of purple bacteria and PSII the identity and structure of the latter could be predicted (Michel and Deisenhofer 1986, Trebst 1987) leading in recent years to schemes as the one shown in Fig. 1.3. Till that time the so called core antenna protein CP47 was thought to be the reaction center of PSII (Nakatani 1983, Nakatani *et al.* 1984, Satoh 1986). After the isolation of a reaction center preparation consisting of just the D1 and D2-proteins and cytochrome b559 and capable of charge separation (Nanba and Satoh 1987) it became clear that the previous reaction center model had been wrong. However, the new model indicated that the D1 protein was a crucial component of PSII which was totally unexpected on the basis of what was known about this protein till then.

In the mid seventies a chloroplast protein of 32 kDa was identified with a turnover rate that was considerably faster than that of any other protein in the chloroplast (Eagles- ham and Ellis 1974, Edelman and Reisfeld 1978). Bedbrook *et al.* (1978) found that the transcription of the gene coding for this protein was light-regulated. Steinback *et al.* (1981) identified this protein as the one binding azido-atrazine and thus as a component of PSII. Because this protein bound several classes of herbicides it had been subjected to extensive research efforts to define the binding site of this protein for herbicides. In the same year it was realized that these herbicides act by displacing Q_B from its binding site (Velthuys 1981, Wraight 1981). Kyle *et al.* (1984b) tried to find a relation between the high turnover rate of this protein and the process responsible for photoinactivation of PSII and known as photoinhibition. It was already known that damage to photosynthesis by excessive light was due to the inactivation of PSII (Critchley 1981), but the mechanism was still unclear. Kyle *et al.* (1984b) showed that photoinhibitory damage is accompanied by loss of herbicide binding sites and in a subsequent paper they showed a correlation
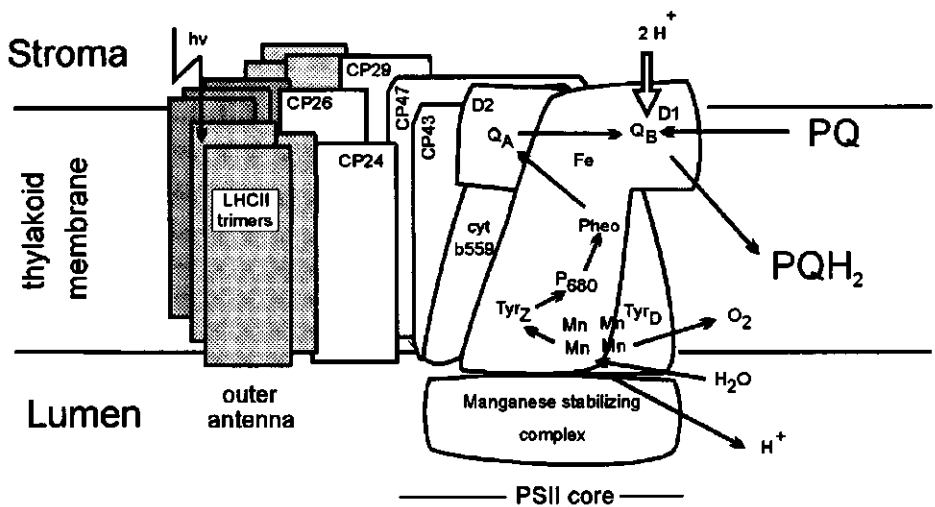
Fig. 1.3. Scheme of PSII. The PSII core consists of the D1 and D2 proteins, a cytochrome b559 molecule, three proteins that form together the manganese stabilizing complex and the core antenna molecules CP47 and CP43. The minor antenna molecules CP24, CP26 and CP29 link the LHCII trimers of the outer antenna to the PSII core. The manganese cluster donates electrons to the PSII reaction center P_{680} via a tyrosine (Tyr_Z). The other electron transport steps are the same as in Fig. 1.1. (Modified from Dau 1994; for more details see Chapter 2.)

between photoinhibitory damage and the loss of the D1 protein (Kyle *et al.* 1985).

The identification of the reaction center of PSII made clear that the D1 protein plays a central role in PSII. Together with the D2 protein, it binds for example almost all the cofactors of PSII involved in
electron transport. A high turnover rate of such an important protein seemed illogical and raised all kinds of questions. The most important one of these which is still not solved is: Why have photosynthetic organisms evolved a mechanism in which they break down and build up such an important protein complex continuously and at a much faster rate than any other protein complex in the chloroplast while for example their purple bacterial ancestors do not? This question focusses attention at two other questions: 1. In which respects differ reaction centers of purple bacteria from PSII reaction centers of higher plants and cyanobacteria? and 2. Has this high turnover of PSII a regulatory function? Another question that is experimentally interwoven with research on photoinhibition is:

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How is PSII and the rest of the electron transport chain regulated on the short, medium and long timescale? In this thesis some aspects of these questions will get attention.

The relative importance of PSII-inactivation to CO_2-fixation

The main target of photoinhibition is PSII. To assess the physiological effect of the inactivation of PSII on CO_2-fixation it is necessary to know which reactions are limiting and in what way this limitation depends on component stoichiometry and irradiance. Evans (1987) has shown, using plants adapted to a range of irradiances, that cyt b_6/f- and ATPase-content correlated reasonably well to changes in the rate of oxygen evolution in leaf discs. No, or a bad correlation was found with the PSI and PSII-content. This finding indicates that under *in vivo* conditions cyt b_6/f limits electron transport, at least when measured in 1% CO_2 and under saturating irradiance. Heber and coworkers (1988) approached this problem in a different way. They partially inhibited several steps in the chain leading to CO_2-fixation and plotted the percent inhibition of electron transport as a function of the percent inhibition of the target enzyme. In this study it was found that PSII controlled electron transport under low irradiances and under uncoupled conditions. Cyt b_6/f controlled electron transport under high irradiances, when the thylakoids are coupled. In intact chloroplasts Calvin cycle enzymes imposed a considerable control over the electron transport rate. Heber *et al.* (1988) found that under coupled conditions and under saturating irradiance up to 40% inhibition of PSII had no effect on the electron transport rate and 85% inhibition of PSII in broken chloroplasts corresponded to 25% inhibition of the electron transport rate using FeCy as an electron acceptor.

Conclusion: *PSII activity is only limiting for the CO_2-fixation rate when the process is not light saturated. At saturating irradiances cytochrome b_6/f becomes limiting.*

Effect of photoinhibition on the rate limiting step

In the literature on photoinhibition a few references concerning the effect of inactivation of PSII on the electron transport rate can be found. Weinbaum *et al.* (1979) observed that in *Spirodela* a 85% depletion of the D1 protein resulted in only 15% inhibition of the CO_2-fixation rate. These results are quite comparable to the ones found by Heber *et al.* (1988). Ögren *et al.* (1984), Walker and Osmond (1986), Björkman *et al.* (1988) working with different plant species, both at normal and low temperatures all observed that the quantum yield for oxygen evolution was more affected by a photoinhibitory treatment than the maximal oxygen evolution rate. Assuming that PSII was affected most by the treatments, these data show that the limiting step shifts from PSII at low irradiances to cyt b_6/f or another component of the chain leading to CO_2-fixation at saturating irradiances.

Long-term adaptation of pea plants to growth irradiance

The photosynthetic properties of pea leaves adapted to different growth irradiances vary (Leong and Anderson 1984a,b, Lee and Whitmarsh 1989). Between 10 and 200 μmol.

m^{-2}.s^{-1} the adaptive response of the photosynthetic apparatus is stronger than at growth irradiances above 200 μmol.m^{-2}.s^{-1}. In plants adapted to a relatively high growth irradiance the Chl b-containing peripheral antennas are smaller in size (higher chl a/chl b ratio). This does not affect the chlorophyll content of the leaves as the PSII content increases (Leong and Anderson 1984a, b). An increase of the Chl a/Chl b ratio was found to be related to a decrease in the extent of stacking (Boardman et al. 1974, Aro et al. 1986, Aro et al. 1994). A high growth irradiance also induces a higher PQ and cyt f content and a higher whole chain electron transport and ATPase activity. In all cases a linear relationship between the Chl a/Chl b ratio and activity or content was observed.

Developing pea leaves are able to adapt their photosynthetic apparatus to the light environment. The ability of mature leaves to adapt to the light environment was studied by analyzing the effects of a sudden increase of the growth irradiance level on the photosynthetic apparatus (Chow and Anderson 1987a,b). It was shown that an increase of the growth irradiance from 60 to 390 μmol.m^{-2}.s^{-1} induced a number of changes in the photosynthetic apparatus of pea chloroplasts. Chow and Anderson (1987b) divided the responses between four groups: 1. an increase of cyt f and of the PQ-pool without an induction-lag-period, 2. a 1-2 days lag period for an increase of the PSII-content and ATPase-activity, 3. a slow but continuous increase in whole chain electron transport activity, and 4. a constant P$_{700}$-content. These changes caused a shift in the PSII:cyt b$_6$/f:PSI-ratio (Chow and Anderson 1987b).

The influence of photoinhibition on photosynthesis
The previous sections show that photosynthetic electron flow is a dynamic process in which the impact of PSII depends strongly on the light environment. Under saturating light many PSII reaction centers can be inactivated without an effect on whole chain activity. Under coupled conditions the electron transport rate is small compared to the rate under uncoupled conditions. On top of that several regulatory mechanisms reduce the efficiency of the antenna of PSII. In other words even under non-photoinhibitory conditions PSII-activity is far from its hypothetical maximum. This overcapacity is necessary because under low light conditions PSII does limit the electron transport rate (Heber et al. 1988). In that respect, the Photosystem II complex is a compromise between differences in demand at low and high light conditions, conditions that a plant under most conditions will encounter daily. Sun plants also have to grow on cloudy days and shadow plants have to be able to survive sun flecks. Even so, photoinhibition affects crop yield and figures of up to 10% loss of yield are mentioned (Ögren and Sjöstrom 1990). In most of these cases the effect of light is aggravated by other stresses like low or high temperatures and drought.

Photoinhibition and physiology
Photoinhibition is a process that is potentially harmful to plant production. A photoinhibi-

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tory treatment is also a tool to study PSII. Excessive light has several specific effects on PSII: a loss of variable fluorescence, a loss of the thermoluminescence B-band, a loss of the Q_A-Fe^{2+}-signal (EPR), and a loss of electron transport between Q_A and the plastoquinone pool. Understanding the photoinactivation of PSII on a mechanistic level yields a lot of information on the physiology of the acceptor side of PSII. Understanding what happens, may also explain why it happens.

This thesis is a continuation of research on the physiology of the acceptor side of PSII that was done at the Department of Plant Physiology of the Wageningen Agricultural University during the last 25 years. This is among others documented in the PhD-theses that were dealing with research on herbicides interacting with PSII (Van Rensen 1971), on bicarbonate (Vermaas 1984, Snel 1985), a molecule that interacts with the Q_B-site and on the mechanism of atrazine resistance (Naber 1989), a mutation that also affects this part of PSII. Atrazine resistant plants were found to be more sensitive to high light (Van Rensen *et al.* 1990) which directed the research interest towards photoinhibition. This thesis adds the effect of photoinhibition on the acceptor side of PSII to the previous themes.

Outline of the thesis
Chapter 2 gives a general description of the photosynthetic machinery, standard experimental methods and of the materials used.

In Chapter 3 a possible role of bicarbonate as a proton shuttle, mediating the protonation of Q_B is discussed. A hypothesis is postulated that the loss of bicarbonate from its binding site is the primary cause of photoinhibition.

Chapter 4 gives a characterization of the electron acceptor SiMo. It was discovered that SiMo can be an important tool to characterize the properties of bicarbonate bound to PSII.

In Chapter 5 differences in the properties of SiMo and FeCy have been used to follow the effects of a photoinhibitory treatment on thylakoids. Especially, differential effects of the pH on both electron transport systems proved to be very informative.

In Chapter 6 the hypothesis postulated in Chapter 3 was approached in a different way. A PSII mutant of *Synechocystis* PCC 6803 with a mutation in the D2 protein close to the bicarbonate binding site and with a higher sensitivity to photoinhibition was characterized in the hope to find a correlation between changed properties of the protonation of plastoquinone and the higher sensitivity to photoinhibition.

Chapter 7 describes an *in vivo* study on the effects of a photoinhibitory treatment on several photoacoustic and fluorescence parameters which gives information on the effects of photoinhibition on the photosynthetic performance of pea leaves.

In Chapter 8 the mechanism of photoinhibition is discussed followed by a discussion of the effects of the inactivation of PSII on the regulation of electron transport.

2 Photosynthetic machinery

Introduction

Though all experimental Chapters have a 'Materials and methods' section, understanding of these chapters presumes much preexisting knowledge. In this chapter information is given on several topics that are important to this thesis. First, electron transport and the properties of electron acceptors used in the Chapters 4, 5 and 6 are discussed. In the second part the interpretation of the fluorescence induction curve and fluorescence decay kinetics are discussed. The third part will focus on the properties of the antenna system of PSII.

Electron transport chain

Localization of the photosynthetic protein complexes

For the transformation of light into NADPH three intrinsic protein complexes are needed: PSII, PSI and cyt b_6/f connected by the electron carriers plastoquinone and plastocyanine. Several studies indicate that PSII is concentrated in the grana stacks and to a lesser extent in the stroma lamellae (Callahan *et al.* 1989, Anderson and Melis 1984, Albertsson *et al.* 1990, Wollenberger *et al.* 1994), cyt b_6/f is more evenly distributed over the different membrane compartments of the chloroplast (Allred and Staehelin 1985, Melis *et al.* 1986, Olive *et al.* 1986, Anderson 1989, Wollenberger *et al.* 1994) and PSI is to be found in the grana margins and in the stroma lamellae (Callahan *et al.* 1989, Albertsson *et al.* 1990, Wollenberger *et al.* 1994). PSII reaction centers in the stroma lamellae (PSIIß) are thought to have properties that deviate from PSII reaction centers in the grana stacks (PSIIα): they have a smaller antenna system (Albertsson *et al.* 1990, Ghirardi *et al.* 1993, Wollenberger *et al.* 1994), are less effective in electron transport (Mäenpää *et al.* 1987, Tyystjärvi *et al.* 1994a) and are less sensitive to photoinhibition (Mäenpää *et al.* 1987, Tyystjärvi *et al.* 1994a). Tyystjärvi *et al.* (1994a) observed that the sensitivity of PSIIß-centers to photoinhibition was only half that of PSIIα-centers. However, they also found that the sensitivity of PSIIα-centers to photoinhibition decreases by a factor 2 if they were

unstacked. This indicates that undoing the lateral separation of PSII and PSI, and thus allowing spillover (Trissl and Wilhelm 1994), protects PSII against photoinhibition (Tyystjärvi et al. 1994a). This notion was supported by the observation that depletion of PSI increased the sensitivity to photoinhibition in unstacked membranes (Tyystjärvi et al. 1994a). The function of the PSIIß-centers is unclear. There are hypotheses that they are intermediates in the synthesis route of PSIIα centers or play a role in cyclic electron transport (Guenther and Melis 1990, Anderson 1989).

Electron and proton transfer
PSII consists of at least 25 different proteins but here we will concentrate on the two proteins that form the reaction center: D1 and D2. These two proteins bind all the prosthetic groups necessary for electron transport and provide most of the ligands for the manganese cluster. The chlorophyll P_{680} uses the light energy absorbed by the antenna to translocate an electron via a pheophytin to the primary quinone acceptor Q_A on the stroma side of the membrane. Q_A transfers this electron to a mobile quinone docked at the Q_B-site forming a semiquinone. After a second charge separation and the uptake of two protons the semiquinone is reduced to a quinol that can leave the Q_B-binding site and diffuse to a nearby cytochrome b_6/f-complex. Work of Joliot et al. (1992) and Lavergne et al. (1992) has shown that several PSII reaction centers and cyt b_6/f-complexes share a plastoquinone pool and that the size of this plastoquinone pool is variable. Plastoquinone/plastoquinol diffusion between pools is hampered by the high protein/lipid ratio. The reoxidation of plastoquinol is a complex process. Understanding of the mechanism of reoxidation has been increasing rapidly in the last few years (Kramer and Crofts 1994, Hope et al. 1994, Huang et al. 1994). The present view incorporating the functioning of a Q-cycle (e.g. Ort 1987) is that one of the electrons donated by plastoquinol is used to reduce plastocyanine and the other is transferred to a plastoquinone binding site on the stroma side of cyt b_6/f (Kramer and Crofts 1994) where in two steps a plastoquinone molecule is reduced to plastoquinol. In this way two extra protons are transferred from the stroma to the lumen improving the ATP/NADPH-ratio. The cyt b_6/f-complex is a functional dimer (the monomeric form is inactive) (Huang et al. 1994). It is unclear whether the two monomers cooperate in the process of reoxidation of plastoquinol or that the interaction between both monomers is only needed to keep cyt b_6/f in a viable conformation (Huang et al. 1994). The reoxidation of plastoquinol is sensitive to the pH in the lumen. According to Hope et al. (1994) the reduction of cyt b563 by the unprotonated semiquinone is the most likely pH-sensitive step. For the oxidation of plastoquinol two protons have to be donated to the lumen and two electrons donated to the cyt b_6/f-complex. The second electron can only be donated if the plastosemiquinone is in the unprotonated form. As the pH in the lumen decreases, the proton concentration increases and the probability that the plastosemiquino-ne is in the unprotonated form decreases. This also means that the probability that the plastosemiquinone can donate an electron to cyt b563 decreases as the pH decreases. For

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the reoxidation of decylquinol a pK-value of 6.1 was found (Hope *et al.* 1994).

Plastocyanine moves to PSI, where it reduces P_{700}^+. Light absorbed by the PSI antenna is used to transfer the electron to the stroma side of PSI. On the acceptor side of PSI the electron is transferred to ferredoxin, which transfers the electron in turn to ferredoxin-NADP oxidoreductase that is able to reduce $NADP^+$.

Electron acceptors and donors
In this thesis several electron acceptors and donors were used: in Chapter 3 SiMo and FeCy, in Chapter 5 also DCPIPH$_2$/ascorbate and MV and in Chapter 6 a quinone (DMBQ). Silicomolybdate will be characterized extensively in Chapter 4 and will not be discussed here. Some properties of the other donors and acceptors will be given below.

Ferricyanide
Ferricyanide is an electron acceptor that under normal circumstances accepts electrons from PSI (on the stroma side). Brewer and Jagendorf (1965) and Wraight (1985) have shown that in thylakoids dark incubated with FeCy for several minutes the electron acceptor is able to oxidize the non-heme iron. Brewer and Jagendorf also observed that oxidation of the non-heme iron did not occur in the presence of light. In other words if PSII is photochemically active FeCy is unable to accept electrons from PSII. This means that under our experimental conditions FeCy accepted electrons from PSI in all cases.

Methylviologen
Methylviologen accepts electrons from the reducing side of PSI. The free radical of MV catalyzes the reduction of oxygen to superoxide. The superoxide radical is protonated to form hydrogen peroxide. In the presence of DTE, hydrogen peroxide takes up two protons of DTE which results in the formation of two water molecules and an S-bridged DTE form. As a consequence there is a net loss of O_2. Contrary to FeCy, MV can only take up electrons from the acceptor side of PSI.

DCPIP and DCPIPH$_2$/ascorbate
Dichlorophenolindophenol is a blue dye that becomes colorless when reduced. DCPIP is a charged molecule and does not easily penetrate the membrane. There are several reports that DCPIP accepts electrons predominantly at the acceptor side of PSI and that the reduction rate at PSII is about 50 times smaller than the reduction rate at PSI (Kok *et al.* 1967, Lien and Bannister 1971, Kimimura and Katoh 1973). Reduced DCPIP is able to move within the thylakoid membrane and donates electrons to the cytochrome b$_6$/f complex and to PSI (Gould and Izawa 1973a, Izawa *et al.* 1973, Gould 1975). The first donation site of DCPIPH$_2$ was shown to be before cytochrome f (Larkum and Bonner 1972) and insensitive to DBMIB (Gould and Izawa 1973b, Houba 1994). Donation of electrons to PSI by DCPIPH$_2$ depends on the integrity of the membrane (Gould and Izawa

1973b). Ascorbate increased oxygen uptake by the system $DCPIPH_2 \rightarrow MV$ considerably (Ort and Izawa 1974). This increase was shown to be due to a reaction between superoxide and ascorbate and could be prevented by the addition of SOD which increases the reduction rate of superoxide to hydrogen peroxide. As $DCPIPH_2$ is oxidized two protons are donated to the lumen creating a pH-gradient between stroma and lumen. The $DCPIPH_2$-oxidation rate is sensitive to the pH in the lumen and this rate is stimulated by the addition of an uncoupler. This characteristic enables the use of $DCPIPH_2$ as a probe for the integrity of the thylakoid membrane. The electron donation rate of $DCPIPH_2$ to both reduction sites (*i.e.* cyt b_6/f and PSI) is sensitive to the pH (Gould 1975) and does not affect the usefulness of this reaction to monitor the integrity of the thylakoid membrane. It has been suggested that the access of reduced DCPIP to its binding sites is pH-dependent (Gould 1975).

Substituted benzoquinones
In Chapter 6 a substituted benzoquinone (DMBQ) has been used that can substitute for the natural occurring quinone. Binding of benzoquinones to PSII was studied extensively in oxygen evolving Photosystem II particles of *Synechococcus* (Satoh *et al.* 1992, 1994). With the exception of duroquinone all benzoquinones tested caused an increase of the binding constant for DCMU. All these benzoquinones are able to bind to the Q_B-binding site and compete with DCMU for this site (Satoh *et al.* 1992). Comparing the binding constants resulted in the following order of binding affinities: DCBQ $>>$ BQ $>$ 2,5-DMBQ $>>$ 2,6-DMBQ. Of the benzoquinones tested only 2,5-DMBQ and duroquinone were not able to oxidize the non-heme iron. The natural occurring quinone also fails to oxidize the non-heme iron (Petrouleas and Diner 1987) but is able to do so if the midpoint potential of the non-heme iron is lowered (Deligiannakis *et al.* 1994). It was observed that DMBQ preferentially accepts electrons from PSI though lowering the pH from 7.5 to 6.0 shifted electron acceptance somewhat to PSII (Kimimura and Katoh 1973). The action of DBMIB (both an inhibitor of the cyt b_6/f complex and an electron acceptor of PSII) is insensitive to KCN; DBMIB accepts electrons directly from PSII (Gould and Izawa 1973). Oxidized quinones are able to quench fluorescence in two ways: by oxidizing Q_A^- and through static quenching of emitted fluorescence. In the past, differences between the abilities of benzoquinones to quench fluorescence statically have not always been separated from differences in their ability to accept electrons from Q_A^-. The ability of DCBQ to reactivate inactive centers (Graan and Ort 1986, Cao and Govindjee 1990, Nedbal *et al.* 1991) was made less likely in a careful study of Lavergne and Leci (1993). The latter authors associated the difference between DCBQ on the one hand and FeCy and DMBQ on the other with differences in the ability of these compounds to keep the plastoquinone pool oxidized.

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Fluorescence induction

The induction curve of fluorescence in the absence of DCMU is a powerful tool to study electron transport and the properties of Photosystem II. A major problem is that there exists no consensus on the interpretation of these curves in mechanistic terms. There are several interpretations of the different phases of the induction curve (Neubauer and Schreiber 1987, Schreiber and Neubauer 1987, Strasser and Govindjee 1991, 1992, Strasser et al. 1995). Neubauer and Schreiber (1987) named the different phases $O-I_1-I_2-P$ and Strasser and Govindjee (1991) O-J-I-P, but the characteristics of the different phases are the same in both cases (Strasser et al. 1995). It was shown that these phases occur both in higher plants and cyanobacteria (Strasser et al. 1995). The I_2-P-phase was assigned to the reduction of the plastoquinone pool (Neubauer and Schreiber 1987). It was also observed that it was impossible to reach the F_m-level in less than 200 ms even at extremely high irradiances. The I_1-level could be reached in about 800 μs, and the fluorescence induced by a saturating single turnover flash, never exceeded the maximal I_1-level (Neubauer and Schreiber 1987). This indicates that the I_1-level represents the state $Q_A^-Q_B$. At saturating photon flux densities (3000 W.m^{-2}) methylviologen was unable to quench the I_2-level. This indicates that the I_2-level represents the state $Q_A^-Q_B^=$. However, at low and moderate photon flux densities electrons are already transferred from Q_A^- to Q_B before all PSII reaction centers have accepted a photon. In Chapter 6 the fluorescence induction curve is used to characterize a mutant of *Synechocystis* PCC 6803. The interpretation of Neubauer and Schreiber (1987) implies that arbitrary equilibria between the different reduction states of Q_A and Q_B are measured at one photon flux density. Therefore, conclusions on the effect of a mutation on electron transfer within PSII can only be extracted from studying the irradiance dependence of the different phases of the induction curve.

Fluorescence decay

It is possible to obtain information from the reoxidation kinetics of Q_A^- after one or two saturating single turnover flashes. Reoxidation of Q_A can occur as a consequence of electron transfer to Q_B or Q_B^- or as a consequence of recombination with one of the S-states on the donor side of PSII. All these processes have different relaxation halftimes and multi-exponential analysis of the decay curve allows the separation of these processes. There is consensus about the interpretation of the fast phases (100-200 μs and 300-600 μs halftimes). These two phases represent electron transfer from Q_A^- to Q_B and Q_A^- to Q_B^- respectively (Crofts et al. 1984, Prasil et al. 1992).

The ms-phase in the decay curve is thought to be associated with the binding of a plastoquinone molecule to an empty Q_B-site (Crofts and Wraight 1983). Another option would be that protonation of double reduced Q_B is the rate limiting step, determining the rate of exchange. It was shown that the halftime of alkalinization of the stroma is about 5 ms in stacked membranes and this halftime decreases after destacking (2.7 ms) and even

more in intermittent-light-grown plants (1 ms) (Jahns and Junge 1992). These halftimes are in the same time domain as the ms-phase of fluorescence decay and indicate that the accessibility of protons to PSII might limit the release of plastoquinol and also the exchange with a plastoquinone molecule.

Electron transfer from Q_A^- to Q_B is 20 times more efficient than the reverse reaction (Diner 1977, Robinson and Crofts 1983). However, this means that at any time 5% of the electrons on the acceptor side of PSII will be localized on Q_A allowing recombination with the donor side of PSII after 1 flash (halftime of several hundreds of ms) even though electron transfer to Q_B is much faster. A shift of the equilibrium between Q_A and Q_B towards Q_A would increase the population of electrons on Q_A and would increase the fraction of Q_A^- recombining with the donor side on a sub-second timescale. This line of reasoning would imply that the fraction of fluorescence that relaxes in more than 50 ms would depend linearly on the probability that an electron will reside on Q_A as a consequence of the equilibrium between Q_A^- and Q_B^-. In herbicide-resistant mutants of *Synechocystis* PCC 6714 a negative correlation was found between the fraction of the slow phase and the temperature of the B-band emission of thermoluminescence (recombination between Q_B^- and S_2) (Etienne *et al.* 1990). This also indicates that there exists a correlation between the fraction of the slow phase and the equilibrium between Q_A^- and Q_B^-.

Antenna system of PSII
The knowledge on PSII antenna systems is increasing rapidly in terms of its biochemistry (composition), biophysics (rate constants of energy transfer, trapping), molecular biology and physiology (xanthophyll cycle, non-photochemical quenching, state transitions, light adaptation). To be able to assess the effects of a photoinhibitory treatment on PSII under-standing of these processes is essential.

Biochemistry
In the last 5 years many data on the protein and pigment composition of antennas have been accumulated due to an improvement in both separation and isolation techniques. With the exception of the xanthophylls there is a consensus about which pigments bind to the different proteins. There is also a good deal of consensus on the components that make up the antenna (*c.f.* Fig. 1.3). There are several reports indicating that PSII is a dimer (Bassi *et al.*, Peter and Thornber 1991, Dainese and Bassi 1991). On the basis of the collected data several antenna models have been proposed (see Jansson 1994 for a review). The core of PSII consists of the reaction center proteins (D1, D2 and cyt b559) and the core antenna proteins CP43 and CP47. The outer antenna consisting of LHCII molecules is linked to the core by means of several proteins named CP24, CP26 and CP29 that seem to function as some kind of linker (Bassi and Dainese 1992). These so

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called minor components have been implicated in the regulation of exciton flow through the antenna. The distribution of the xanthophylls over the antenna proteins is still a matter of debate. The minor components seem to be enriched in these pigments. However, there is still uncertainty about the xanthophyll content of the LHCII molecules (Peter and Thornber 1991, Bassi *et al.* 1993, Ruban *et al.* 1994) and to what extent the different pools of violaxanthins can be transformed into zeaxanthins (Bassi *et al.* 1993, Ruban *et al.* 1994).

Biophysics

Schatz *et al.* (1986) hypothesized that PSII is trap-limited and that it is a shallow trap. This means that equilibration of an exciton within the antenna is a much faster process than the stabilization of charge separation (trapping). In other words, an exciton can move in and out of the reaction center several times before it is trapped. The shallowness of the trap also implies that there is a considerable probability to find an exciton outside of the reaction center. When looking at the distribution of excitons over the antenna the following numbers were reported: reaction center 5%, core antenna 30%, minor antenna components 16% and the light harvesting complex 49%. If the relative size of each of these components is taken into account a slight concentration of excitons in the core of PSII is found (Jennings *et al.* 1992). When trapping in PSII and PSI is compared it is found that PSI traps excitons about 3 times more efficient than PSII (Table 2.1). The data in Table 2.1 indicate that the life time of an exciton in PSI is much shorter than in PSII and that reduction of Q_A increases the lifetime about 6-fold. The longer the lifetime of an exciton the greater the probability for a quenching event to occur. Another advantage of the shallow trap and the concomitant small probability of finding an exciton on P_{680} is that the probability of the production of triplet chlorophylls and singlet oxygen is also small.

Table 2.1. Lifetimes (τ) of excitons in open and closed reaction centers of PSII and PSI in cells of Scenedesmus obliquus. The data were derived from Lee et al. (1990) and Holzwarth (1990). Closing PSI had no effect on the lifetime of an exciton in the antenna of PSI.

	Open reaction centers	Closed reaction centers
PSII	τ = 440-550 ps	τ = 1.5 ns
PSI	τ = 94-96 ps	τ = 114-118 ps

LHCII-phosphorylation

There are several processes associated with the antenna that influence exciton migration within the antenna. LHCII is phosphorylated when PSII receives more light than PSI, a

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process regulated by the redox state of the cyt b_6/f-complex (Bennett 1991, Allen 1992, see also Fig. 1.2). If the cyt b_6/f-complex is in the reduced state the kinase that phosphorylates LHCII is activated and if the cyt b_6/f complex is oxidized this kinase is inactivated. It was found that the kinase needed for the phosphorylation is inhibited during a photoinhibitory treatment (Schuster *et al.* 1986, Demmig *et al.* 1987, Cleland *et al.* 1990, Ebbert and Godde 1994). As a consequence of the phosphorylation of LHCII a part of the outer antenna disconnects from PSII. A smaller antenna size of PSII relieves the excitation pressure on PSII. What happens to the disconnected LHCII is unclear. Some experiments seem to indicate that phosphorylated LHCII migrates from the grana to the stroma (Kyle *et al.* 1984a, Larsson and Andersson 1985). However, recent experiments indicate that one has to be careful with this interpretation. Georgakopoulos and Argyoudi-Akoyunoglou (1994) observed an LHCII enrichment in the stroma lamellae fraction after LHCII phosphorylation. However, they also showed that this stroma fraction was not homogeneous and could be separated in an LHCII-rich and an LHCII-poor fraction. The LHCII-rich fraction had properties intermediate between grana and stroma lamellae. A membrane fraction with comparable properties could be isolated from isolated grana stacks after a phosphorylation treatment. These data indicate that phosphorylated LHCII did not migrate to the stroma lamellae but could be found in unstacked grana membranes. Kohorn and Yakir (1990), studying import of p(recursor)LHCII, showed in a very elegant way that phosphorylation of LHCII had no effect on the migration pattern of LHCII. Both pLHCII containing a phosphorylation site as well as pLHCII without this site showed the same migration pattern, both under phosphorylating and non-phosphorylating conditions. Under all experimental circumstances an unidirectional migration from stroma to grana was observed. The only difference was that pLHCIIs without a phosphorylation site were more sensitive to degradation. As an alternative hypothesis Kohorn and Yakir (1990) referred to a theory postulated by Glazer and Melis (1987) that phosphorylation marks LHCII for degradation. Although these two papers make connection of phosphorylated LHCII to PSI in the stroma less likely, connection to PSI in the grana margins is still possible. If connection of phosphorylated LHCII to PSI occurs, its efficiency is low (Larsson *et al.* 1986).

Connectivity

If connectivity between antenna systems of reaction centers exists, a photoinhibitory treatment should affect several fluorescence parameters relative to the values before the photoinhibitory treatment. Connectivity means that excitons in the antenna system of an inactivated reaction center can be transferred to systems with still active reaction centers. If the inactivation of PSII is a random process, 50% inactivation makes a double number of excitons available to the still active reaction centers causing a decrease of the number of open reaction centers at a given photon flux density, in other words a decrease of photochemical quenching (q_P). This effect would be partially counterbalanced by a

stronger demand of PSI for the electrons of the still active PSII reaction centers. The effect of connectivity would be further modulated by the effects of state transitions and changes in the outer antenna associated with the formation of zeaxanthin. All these complications make it often difficult to determine if connectivity occurs and to what extent (*e.g.* Chapter 7).

If connectivity occurs, photoinhibited thylakoids, in which the xanthophyll cycle and LHCII phosphorylation are impaired, are expected to be affected most by connectivity. Cleland *et al.* (1986) tried to demonstrate the existence of connectivity in spinach thylakoids by determining the rate constant K_α of Q_A-reduction in the presence of DCMU in samples in which 50% of the reaction centers were inactivated by a photoinhibitory treatment. In these samples the rate constant of Q_A-reduction increased by a factor 1.2 ± 0.1. If all inactivated PSII reaction centers had donated their excitons to still active reaction centers the rate constant of Q_A-reduction should have increased by a factor 2. The observed increase is relatively marginal compared to the maximal effect. These data indicate that even under optimal conditions for connectivity its role is marginal.

One of the main sources of evidence to support connectivity is the sigmoid shape of the fluorescence induction curve in the presence of DCMU (Joliot and Joliot 1964). Trissl *et al.* (1993) and Trissl and Lavergne (1994) evaluated the fluorescence induction curve on the basis of the exciton-radical pair model. In the first paper it was concluded that it is not possible to use the form of the sigmoid curve to determine the extent of connectivity. However, in the second paper this conclusion was modified and it was concluded that fluorescence induction curves indicate that there is limited exciton transfer between PSII reaction centers. One of the models that was suggested to explain the data was a limited energy transfer between PSII dimers. The existence of connectivity should also influence the amount of oxygen produced by a flash of light. If connectivity is effective relatively more oxygen should be produced on a flash if part of the reaction centers are closed by background illumination than in a fully oxidized system. Ley and Mauzerall (1986) performed this experiment and were not able to find any proof for the occurrence of connectivity in *Chlorella*. According to these authors transfer of excitons between different antennas has little effect on the effective antenna size as the probability that an exciton escapes from an open PSII is about the same as the probability that it escapes from a closed PSII.

3 Hypothesis

The irreversible release of bicarbonate is the trigger for the inactivation of Photosystem II under photoinhibitory conditions

Summary

A hypothesis is formulated in which bicarbonate/CO_2 bound to PSII plays a role in the protonation of Q_B. The acceptor side of PSII functions as a carbonic anhydrase in which bicarbonate/CO_2 as well as the non-heme iron plays a role. Photosystem II is inactivated when bicarbonate/CO_2 is released from its binding site; the probability that this occurs is higher at higher irradiance levels.

Introduction

The D1 protein is one of the central components of PSII (see Chapter 1). The turnover rate of this protein is higher than that of any other protein in the chloroplast (Edelman and Reisfeld 1978). The D1-turnover rate is light dependent and occurs already at low irradiance levels (Mattoo *et al.* 1984, Greenberg *et al.* 1989b, Aro *et al.* 1993, Sundby *et al.* 1993). A continuous high synthesis rate of this abundant protein seems expensive in terms of the consumption of ATP. Photosynthetic organisms apparently have good reasons to maintain such an expensive process. Attempts to elucidate these reasons have not yet been successful.

Turnover of the D1 protein can be seen as a regulatory mechanism (Öquist *et al.* 1992b) or as a 'flaw' in the architecture of PSII (Barber and Andersson 1992). The second option allows an analysis of the functional weaknesses of PSII. There are several observations on photoinhibition related to D1 turnover. In bacterial reaction centers, a

high turnover of reaction center components has not been observed (Prasil *et al.* 1992). This draws the attention to differences between bacterial reaction centers and PSII reaction centers. Barber and Andersson (1992) concentrated in their analysis of the differences between bacterial reaction centers and PSII reaction centers on the very high oxidation-reduction potential ($E_{m,7}$) of P_{680}^+ ($+1.1$ V) relative to a potential of about $+0.5$ V in the reaction centers of purple bacteria and on the fact that O_2 (a reactive agent) is a byproduct of water splitting whereas purple bacteria use other substrates (e.g., H_2S) as a hydrogen donor. However, PSII reaction centers have found ways to cope with the risks of deleterious reactions with P_{680}^+ (see Chapter 2 for a discussion). The suggestion that photoinhibition is caused by oxygen radicals produced on the donor side is contradicted by the observation that *in vivo* the primary lesion of photoinhibition is found on the acceptor side of PSII (Kirilovsky *et al.* 1988, 1990, Briantais *et al.* 1992, Zer *et al.* 1994) instead of on the lumen side. There are not only differences between the donor side of bacterial reaction centers and of PSII reaction centers but also on the acceptor side. The non-heme iron that is thought to be inert in bacterial reaction centers is redox active in PSII reaction centers. In contrast to bacterial reaction centers, PSII reaction centers bind a bicarbonate or CO_2 molecule. In photosynthetic bacteria protonation of Q_B takes place by a "bucket brigade" of amino acid residues that hand over the proton to Q_B (e.g. Okamura and Feher 1992, McPherson *et al.* 1994). However, this system is only effective as long as the length of the "bucket brigade" is limited (Paddock pers. comm.). It has been suggested by several authors that CO_2 or bicarbonate plays an important role in the protonation of Q_B (Eaton Rye 1987, Van Rensen *et al.* 1988, Blubaugh and Govindjee 1988). Bicarbonate could for example shuttle protons between the stroma and the Q_B molecule that is buried deeply in the protein matrix. This would imply that during the evolution from a bacterial reaction center towards a PSII reaction center, the mechanism of Q_B protonation has changed. Two possible reasons for this change might be that: 1. PSII is a considerably more complex protein complex than the bacterial reaction center. One possible consequence of this increased complexity might be that the "bucket brigade" is no longer effective in PSII reaction centers. 2. The presence of a diffusible component (bicarbonate/CO_2) that is indispensable for PSII activity allows regulation of PSII.

Hypothesis
On the basis of the data presented in this introduction we have formulated the following hypothesis:
Photosystem II uses bicarbonate and the non-heme iron to make Q_B accessible to protons from the stroma. The role of the non-heme iron in the protonation of Q_B is that it catalyses the hydration of CO_2 which makes a proton available to reduced Q_B (Fig. 3.1). CO_2 is a diffusible molecule and there is a certain probability that this CO_2 molecule diffuses out of PSII. The probability that bicarbonate/CO_2 can diffuse out of PSII increases as the irradiance increases or to put it differently, this probability depends on

the light dose. This loss is irreversible (Bowden *et al.* 1991). Loss of bicarbonate/CO_2 inhibits the protonation of reduced Q_B inhibiting electron transport. It is predicted that in BBY-particles or PSII-membranes that lack part of the outer antenna this irreversibility is less or the effect of the loss of CO_2 on PSII activity is less drastic because protons have easier access to the Q_B site.

The loss of bicarbonate/CO_2 may cause a conformational change and may make the D1 protein a substrate for a protease leading to D1 degradation. Another possibility might be that the loss of bicarbonate/CO_2 allows O_2 to become a ligand to the non-heme iron. This molecule might cause oxidative damage to the protein environment of the non-heme iron and this process might also make the D1 protein a substrate for a protease.

Proposed mechanism for a role of bicarbonate in the protonation of Q_B

If it is assumed that bicarbonate/CO_2 functions as a proton shuttle, the first question is: where does the proton originate from. There are two possibilities: 1. HCO_3^- donates an proton as suggested by Eaton Rye (1987). The pK_a value of this reaction in an aqueous solution is 10.2 (Blubaugh and Govindjee 1988). Because this pK_a value is rather basic, even if the protein environment is able to shift it to lower values, this reaction is unlikely. 2. H_2CO_3 donates a proton. The pK_a value of this reaction is 6.4. Crofts *et al.* (1984) studied the pH dependence of the reduction of Q_B and concluded that a group within PSII was protonated and that the pK of this reaction was 6.4. The pK of this protonatable group, possibly a histidine (Blubaugh and Govindjee 1988), shifts to 7.9 upon the formation of Q_B^- (Crofts *et al.* 1984). The pK_a value of this protonation reaction is close to the pK_a value of the deprotonation of H_2CO_3. H_2CO_3 exists in an equilibrium with CO_2 and H_2O. If PSII has carbonic anhydrase activity and operates with a reaction mechanism that is comparable to that of carbonic anhydrase (see Liljas *et al.* 1994) the reaction scheme would be as depicted in Fig. 3.1: In the starting situation, a bicarbonate molecule is close to the stroma, a water molecule is bound to the non-heme iron and Q_A and Q_B are in the oxidized state. Bicarbonate accepts a proton from a carboxyl group close to the stroma and dissociates into H_2O and CO_2 (see step 1). In step 2, a charge separation takes place and Q_B becomes reduced. The negative charge on Q_B and the presence of the non-heme iron facilitate the deprotonation of the H_2O molecule bound to the non-heme iron and the association of this proton with one of the histidine ligands. The carboxyl group close to the stroma becomes protonated again by a proton from the stroma. In step 3, the CO_2 molecule reacts with the hydroxyl group bound to the non-heme iron and a bicarbonate molecule is formed. In step 4, a second charge separation takes place. The negative charge on Q_A facilitates the displacement of the bicarbonate molecule at the non-heme iron for a H_2O molecule making the system ready for the second protonation cycle.

There are two observations that support this scheme: 1. In purple bacteria water molecules were observed close to the non-heme iron (Deisenhofer and Michel 1989, Michel 1991, Okamura and Feher 1992); thus, the possibility of bound water in PSII is
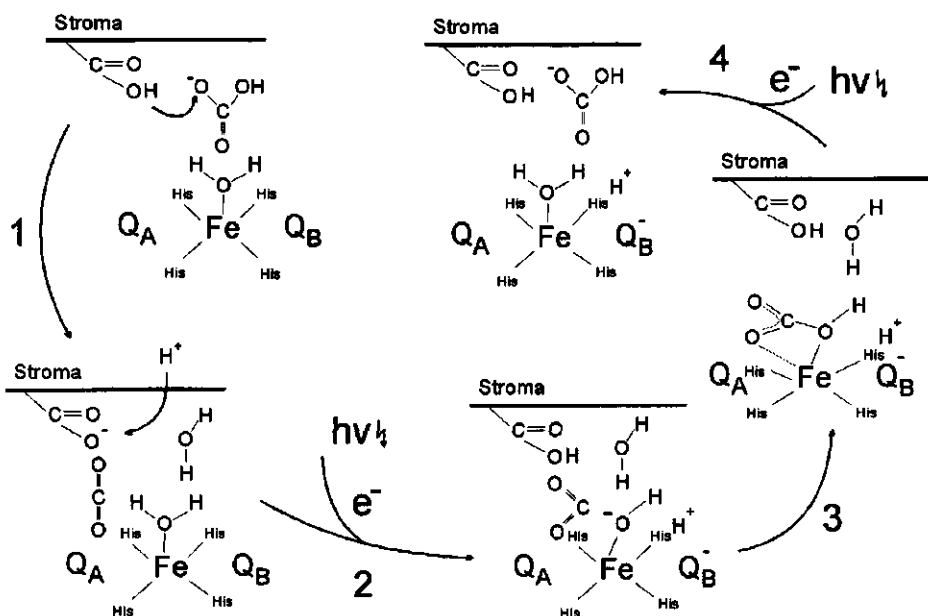
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Fig. 3.1. Proposed scheme for a proton shuttle activity of bicarbonate/CO₂ based on the mechanism of carbonic anhydrase (Liljas et al. 1994). See text for further details.

feasible and 2. Indications were found that the two non-histidine ligands of the non-heme iron are two chemically different molecules (V. Petrouleas and coworkers pers. comm.)

Bicarbonate binding to PSII

Bicarbonate/CO_2 binding is known for quite some time to affect the acceptor side of PSII drastically. The presence of bicarbonate on the acceptor side of PSII can be derived from several experiments. For example, addition of bicarbonate is able to reverse the inhibition of PSII by a range of anions (formate, fluoride, nitric oxide (NO), acetate, glyoxylate, etc.) (Stemler and Murphy 1985, Diner and Petrouleas 1990, Deligiannakis et al. 1994). Fluorescence studies have shown that the inhibitory site of these anions is located at the acceptor side of PSII (Govindjee et al. 1976). Displacement of bicarbonate by formate induces changes in the EPR-signal (Vermaas and Rutherford 1984). Two results were reported that indicate that these changes are caused by the displacement of bicarbonate and not ᵇʸ the binding of formate: 1. Bowden et al. (1991) observed that comparable

changes in the EPR spectrum take place during the isolation of PSII membranes. These changes can be prevented by performing the isolation procedure in the presence of bicarbonate, and 2. Diner and Petrouleas (1990) observed that bicarbonate/CO_2 displacement by formate and NO, respectively, had the same effect on the EPR spectrum even though these two molecules have separate binding sites. Another result that supports bicarbonate/CO_2 binding to PSII was reported by Govindjee *et al.* (1991) who showed, using an infrared gas analyzer, that CO_2 release was measurable after the addition of formate. The presence of bicarbonate/CO_2 is also supported by SiMo binding studies. Silicomolybdate displaces labeled bicarbonate/CO_2 (Stemler 1977) and the pH dependence of SiMo binding is best explained by assuming the presence of bicarbonate/CO_2 on the acceptor side of PSII (Schansker and Van Rensen 1993).

Characteristics of bicarbonate binding
Stemler and coworkers (1977, 1980, 1983, 1984) have investigated the binding characteristics of bicarbonate to PSII. First the effect of the pH on the binding of labeled bicarbonate to CO_2-depleted thylakoids was studied (Stemler 1977). The rate of bicarbonate binding was 10-fold higher at pH 6.0 than at pH 7.8. However, using non-CO_2-depleted thylakoids, exchange experiments with labeled bicarbonate (Stemler and Murphy 1983) and with NO (Diner and Petrouleas 1990) indicated that above pH 7.0 almost no exchange of bound bicarbonate with the stroma occurs.

An interesting feature is the role of light in the binding process. Stemler (1979) found that thylakoids depleted of bicarbonate with 100 mM formate can only be reactivated by addition of bicarbonate in the dark. The presence of light prevents reactivation. Further studies in which preflashes were given indicated that if one single turnover flash was given to bicarbonate-depleted thylakoids kept in the dark reactivation by bicarbonate was inhibited more than after no or two preflashes. This was taken as evidence that binding of bicarbonate to PSII is optimal when the bound plastoquinone is in the oxidized state. Stemler and Murphy (1983) showed that the presence or absence of formate plays an important role in this effect. In the absence of formate normal room light lowered the binding affinity of bicarbonate from 42 μM to 56 μM. In the presence of 20 mM formate the binding affinity decreased from 148 μM in darkness to 480 μM in room light. That bicarbonate can be more easily exchanged for formate in the light was also observed by Vermaas and Van Rensen (1981) and Van Rensen *et al.* (1988). The non-heme iron cannot be oxidized by the natural quinone (Zimmerman and Rutherford 1986, Diner and Petrouleas 1987) and the very fast reoxidation of Q_A^- by the oxidized non-heme iron (25 μs) is only observed after a pretreatment with FeCy (Diner and Petrouleas 1987, Diner *et al.* 1991). Under normal conditions, the non-heme iron is in the reduced state. This raises the question about the reason for the differences in the binding of bicarbonate in the dark from that in the light. One difference might be that in the light bicarbonate/CO_2 is not firmly bound to the non-heme iron and it can escape only in the

unbound state to the stroma.

The non-heme iron and bicarbonate binding

Contrary to the non-heme iron of PSII reaction centers, the bacterial one is chemically inert and extraction or substitution by other divalent metal ions does not inactivate the reaction center (Debus *et al.* 1986). In PSII reaction centers of higher plants, extraction or substitution is not possible without total inactivation of the reaction center (Akabori *et al.* 1992). Redox activity of the non-heme iron has only been observed using artificial quinones like DCBQ or PpBQ, the midpoint potential of the native quinone is not high enough to oxidize the non-heme iron (Zimmerman and Rutherford 1986, Diner and Petrouleas 1987, Deligiannakis *et al.* 1994). The non-heme iron has six ligands. Four of them are histidines, the two remaining ligands are still unclear. A bidentate binding of bicarbonate to the non-heme iron has been suggested to form the 5th and the 6th ligand to the non-heme iron (Michel and Deisenhofer 1988, Blubaugh and Govindjee 1988). It has been reported that the 5th and the 6th ligand are chemically different (V. Petrouleas and coworkers pers. comm.). In Fig. 3.1 a mix of both options is assumed.

Carbonic anhydrase activity of PSII

Strong similarities exist between carbonic anhydrase (that catalyzes the hydration of CO_2) and the acceptor side of PSII (Stemler and Jursinic 1983, Stemler 1986). Carbonic anhydrase activity has been observed in thylakoids and PSII particles (Vaklinova *et al.* 1982 and 1984 (quoted by Stemler 1986), Stemler 1986, Pronina and Semenenko 1988, Stemler and Jursinic 1993 and Moubarak-Milad and Stemler 1994). The carbonic anhydrase activity can be inhibited by the same anions that inhibit the acceptor side of PSII: it is inhibited by SiMo and by light (Stemler 1986). The inhibition by light was a gradual and irreversible process (Stemler 1986) and might have been associated with photoinhibition of the sample. The data suggest that the carbonic anhydrase activity is in some way associated with the functional integrity of PSII.

Could this observed carbonic anhydrase activity be the same as the carbonic anhydrase activity of the acceptor side of PSII as proposed in Fig. 3.1 (step 3)?
There are several relevant points in this respect:
1.Moubarak-Milad and Stemler (1994) made redox titrations of binding of labeled bicarbonate to PSII and of carbonic anhydrase activity at pH 6.5 and observed in both cases a midpoint potential of +480 mV. The midpoint potential of carbonic anhydrase activity was pH-dependent (64 mV/pH unit). This value was comparable to a redox titration of formate binding to PSII (midpoint potential of +480 mV at pH 7.0) (Stemler and Jursinic 1993). This value is higher than that of the non-heme iron (400 mV at pH 7.0) Instead of the non-heme iron a donor side component was proposed as the binding component (Stemler and Jursinic 1993). On the basis of the similarities of midpoint

potentials it was inferred that this donor side component was also involved in carbonic anhydrase activity. However, it has been noted (Deligiannakis *et al.* 1994) that formate may affect the midpoint potential of the non-heme iron and that the increased midpoint potential can also be explained by a formate-induced increase of the midpoint potential of the non-heme iron.

2. The similarity between the midpoint potentials of carbonic anhydrase and bicarbonate binding (Milad-Moubarak and Stemler 1994) suggests the involvement of the acceptor side of PSII.

3. Treatments that inhibit PSII also inhibit carbonic anhydrase activity (Stemler and Jursinic 1983, Stemler 1986).

4. Milad-Moubarak and Stemler could still observe carbonic anhydrase activity at pH 7.6, a pH-value at which PSII bound bicarbonate is no longer exchanged with the stroma (Stemler and Murphy 1983). This argues against the conclusion that their observed carbonic anhydrase activity and our proposed PSII acceptor side carbonic anhydrase activity are the same. Thus, the carbonic anhydrase activity reported by Stemler and coworkers is possibly different from the carbonic anhydrase activity proposed in Fig. 3.1.

Distance between stroma and Q_B

Photosystem II has not yet been crystallized. Therefore, all data on the distance between Q_B and the stroma are circumstantial. An observation indicating that a proton has to travel a longer distance in PSII than in bacterial reaction centers to reach Q_B was reported by Jahns and Junge (1992). They found that in intermittent light grown plants (lacking LHCII) proton uptake on the acceptor side at pH 7.0 was much faster (1.1 ms halftime of uptake) than in normal unstacked (2.7 ms) or stacked thylakoids (5 ms). At pH 8.0 the halftime for proton uptake was even close to the electron transfer time from Q_A^- to Q_B^- (600 μs versus 300 - 500 μs). Jahns and Junge (1992) suggested that LHCII proteins shield the protonation site around Q_B, which would increase the distance between stroma and Q_B.

Photoinhibition and bicarbonate release

During a photoinhibitory treatment the Q_A-Fe EPR signal declines in parallel to the inactivation of oxygen evolution (Andersson *et al.* 1989, Van Mieghem *et al.* 1989, Van Wijk *et al.* 1992). This observation was interpreted as an inactivation of the charge stabilization by Q_A and was used as an argument to support the Q_A double-reduction theory (see also Chapter 8). Changes in the environment of the non-heme iron will also affect the Q_A-Fe EPR signal (Ono *et al.* 1995) and effects of an aerobic photoinhibitory treatment on the non-heme iron have been reported (Gleiter *et al.* 1992, Haag *et al.* 1992, Vass *et al.* 1995). Vass *et al.* (1995) observed that in the early stages of the photoinhibitory treatment, part of the changed properties of the non-hem iron can be restored by adding glycolate, a compound that lowers the redox potential of the non-heme

iron. This indicates that the photoinhibitory treatment increases the redox potential of the non-heme iron. In later stages of the photoinhibitory treatment, a growing population of PSII reaction centers is observed in which the glycolate treatment is no longer effective in reversing some of the effects of the photoinhibitory treatment. Vass *et al.* suggest that the modification of the non-heme iron by photoinhibition may be due to a modification in bicarbonate, which might also mean that the bicarbonate is lost as postulated in this Chapter.

Sundby (Sundby 1990 and Sundby *et al.* 1992) tried to prove that bicarbonate plays a role in the mechanism of photoinhibition by studying if added bicarbonate protected thylakoids against photoinhibition. We were not able to reproduce her results. However, there are other reasons to doubt if this approach is useful. Nitric oxide (Diner and Petrouleas 1990) and SiMo (Schansker and Van Rensen 1993) displace bicarbonate in a non-competitive way. This indicates that the addition of bicarbonate may delay the release of bicarbonate somewhat, but it cannot prevent it.

Tools to test the hypothesis that bicarbonate release is the primary event leading to photoinhibition on a thylakoid level or in intact systems are lacking at the moment. In the following chapters several attempts are made to find such tools.

4 Characterization of the complex interaction between the electron acceptor silicomolybdate and Photosystem II

Abstract

Silicomolybdate (SiMo) and its effects have been characterized to evaluate its use as a probe for photosystem II (PS II). It can accept electrons at two places in the electron transport chain: one at PS II and the other at PS I. In the presence of 1 μM 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) only the site at PS II is available. It is suggested that SiMo must displace bicarbonate from its binding site to be able to function as an electron acceptor. This displacement is non-competitive. The binding of SiMo is differentially inhibited by PS II inhibitors: dinoseb > ioxynil > diuron. This difference is determined by the different positions of the inhibitors within the Q_B binding niche and their interaction with bicarbonate. The experimental results show that the SiMo-binding site is not the same as the Q_B-binding site. Analysis of the results indicates that the SiMo-binding niche is located between the parallel helices of the D1 and D2 proteins of PS II close to the non-heme iron. It is concluded that SiMo is an electron acceptor with unique characteristics useful as a probe of the acceptor side of PS II.

Introduction

Girault and Galmiche (1974) introduced silicotungstate as a compound influencing photosynthetic electron transport. Silicotungstate enabled electron transport to FeCy in the presence of DCMU. Since electron transport to dichlorophenolindophenol or methylviologen was not observed in the presence of DCMU and SiTu, the authors concluded that SiTu changed the membrane properties of the thylakoid membrane in such a way that FeCy could accept electrons at a place before the DCMU block and, thus, this electron transport was independent of DCMU. The action of an analogue of silicotungstate, silicomolybdate (SiMo) was studied by Giaquinta and Dilley (1975) and

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Zilinskas and Govindjee (1975) who concluded that SiMo itself was an electron acceptor. Chlorophyll *a* fluorescence measurements (Zilinskas and Govindjee 1975) indicated that SiMo (as well as SiTu) could accept electrons from the primary one-electron acceptor Q_A. Böger (1982) presented data casting doubt on the concept that SiMo accepts electrons from Q_A independently of DCMU since he found interactions between SiMo and DCMU. Using labeled herbicides, Böger contradicted the conclusion of Girault and Galmiche (1974) that SiTu did not displace DCMU. Böger found a clear concentration dependent displacement of DCMU by SiMo. The results of Böger (1982) were confirmed by Graan (1986) who claimed that SiMo occupied the Q_B-binding site just like quinones and because it was a poor electron acceptor there was no reason to use it as a tool in PS II research (also see Critchley 1988). Thus, there has been a decline in the interest in this compound and over the last years few papers on SiMo were published.

In this paper we present a thorough characterization of the interaction of SiMo with PS II. Although we confirm the results of Böger (1982) and Graan (1986), we show that SiMo can be a valuable probe for the acceptor side of PS II and that it is especially useful in the study of the role of bicarbonate in PS II (see Blubaugh and Govindjee 1988, and Van Rensen 1992 for reviews on the bicarbonate effect).

Materials and Methods

Isolation of thylakoid membranes
Pea (*Pisum sativum*) and *Chenopodium album* leaves (triazine sensitive and triazine resistant) of vegetative plants were used to isolate thylakoids, as described elsewhere (Naber 1989). The thylakoids were resuspended in 2 ml isolation medium consisting of 0.4 M sorbitol, 20 mM tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$ and 2 mg.ml^{-1} bovine serum albumin. The chlorophyll (Chl) concentration was determined by the method of Bruinsma (1963). Thylakoids (2 mg Chl.ml^{-1}) were stored at -80 °C until use.

Electron transport measurements
Experiments were conducted at a Chl concentration of 25 μg.ml^{-1} and a temperature of 25 °C. White light of about 5700 μmol.m^{-2}.s^{-1} irradiance was provided by a slide projector. The reaction medium consisted of 50 mM tricine (pH 7.6), 0.3 M sorbitol, 5 mM $MgCl_2$ and 5 mM NH_4Cl. In the experiments in which the pH was 6.3, 50 mM MES was used instead of tricine. Electron transport was measured as oxygen evolution using a Gilson oxygraph as described earlier (Van Rensen *et al.* 1977). In this study we have used 0.1 mM SiMo in most of the experiments. This concentration was not saturating if SiMo was added directly after turning on the light. This means that if SiMo mediated activity is measured directly after addition of 0.1 mM SiMo, SiMo is bound to only part of the PS II centers. The rest of the PS II centers can still transfer electrons to PS I. We used this characteristic to measure the time dependent displacement of bicarbonate by SiMo in Fig.

4.2. It also means that under these circumstances there does not exist an binding equilibrium.

SiMo preparation
SiMo (M_r = 1970) was dissolved in water. The insoluble fraction was removed by centrifugation and the concentration was adjusted using a molar extinction coefficient of $\epsilon400 = 1.07$ cm^{-1}.mM^{-1} (Strickland 1952).

Results

Since its introduction, SiMo has almost always been used in combination with DCMU. As there is an interaction between SiMo and DCMU, studying SiMo in the presence of DCMU complicates its characterization. To avoid this problem we have minimized the use of DCMU.

Instability of SiMo
A complication when using SiMo is its instability at neutral and alkaline pH. Schansker and Van Rensen (1992) reported what happens to SiMo at pH 7.6 by monitoring its absorbance at 400 nm. Within an hour, 50% of the absorbance was lost. In Fig. 4.1, SiMo was incubated for 20 - 22 hours at different pHs. The stability is clearly pH dependent. As can be seen in Fig. 4.1 phtalic acid, used between pH 4.0 and 5.5, stabilizes SiMo. That several other compounds are able to stabilize SiMo is known (Tsigdinos and Hallada 1974). Izawa and Berg (1976) found that 5% glycerol and 1% DMSO can stabilize SiMo. SiMo dissolved in water has a pH below 2. As can be seen in Fig. 4.1, this means that the SiMo stock solution at this low pH is stable for more than a day.

Action at two sites
More important is the fate of SiMo when it is added to a thylakoid suspension. In Fig. 4.2, the rate of oxygen evolution of thylakoids (pH 7.6) is plotted against the time of dark incubation with SiMo. This has been done in the presence and absence of 1 μM DBMIB. DBMIB is known to block the reoxidation of plastoquinol at the used concentration (Trebst *et al.* 1970, Oettmeier *et al.* 1987a). The activity up till 1 min dark incubation is much higher in the absence of 1 μM DBMIB than in its presence. This indicates that SiMo can accept electrons at two sites in the electron transport chain: before the DBMIB-block, and past the DBMIB-block. A second characteristic is the fast decline of the activity that can be attributed to the acceptor site past the DBMIB block. During the first 15 seconds 75% of this PS I mediated activity is abolished and after 2 minutes there is no

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activity left. Compared with the rate of absorbance loss at 400 nm (Schansker and Van
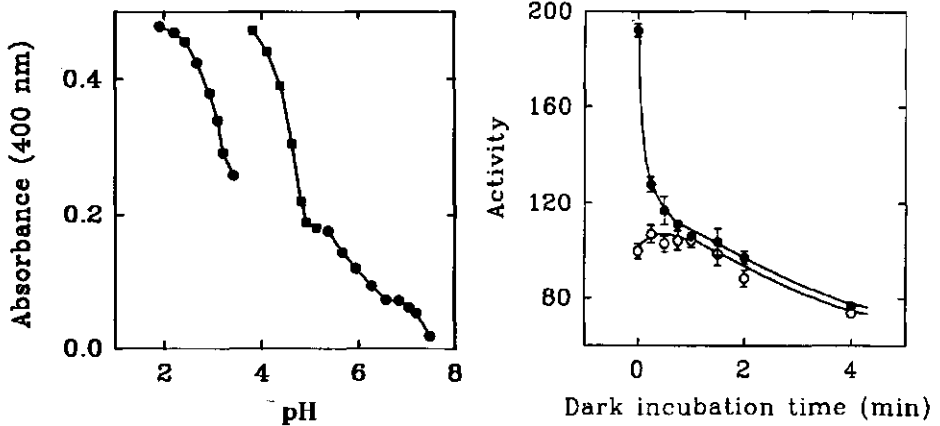

Fig. 4.1. Relation between pH and stability of SiMo. SiMo solutions were kept in the refrigerator for 20-22 hours at different pHs. After this period of time its stability was measured as the decline of absorbance at 400 nm. (filled circles) phosphate buffer; (filled squares) phtalic acid buffer.

Fig. 4.2. Loss of SiMo-mediated electron transport activity as a function of the dark incubation time with SiMo. (closed circles) -DBMIB; (open circles) +1 μM DBMIB. The initial rate of oxygen evolution expressed in $\mu mol(O_2).mg^{-1}(Chl).h^{-1}$ was measured at pH 7.6.

Rensen 1992), the decline of electron transport to PS I is much faster and because there is no comparable decline of activity in the presence of DBMIB this decline is not due to the instability of SiMo. In the presence of DBMIB the activity also declines. However this decline is much more gradual and is much more comparable to the loss of absorbance at 400 nm.

Interaction of herbicides with SiMo
As noted in the Introduction, it was assumed earlier that the electron donation of PS II to SiMo is DCMU insensitive. However, Böger (1982) and Graan (1986) found a strong interaction between SiMo and DCMU.

Graan (1986), using labeled DCMU, determined that at pH 6.5, 15 μM SiMO was needed to decrease DCMU-binding by 50%. In Fig. 4.3, two DCMU-titration curves are shown: one with FeCy as electron acceptor and the other with SiMo. Even 20 μM

Fig. 4.3. DCMU titration curves. (open circles) SiMo as electron acceptor; (filled circles)
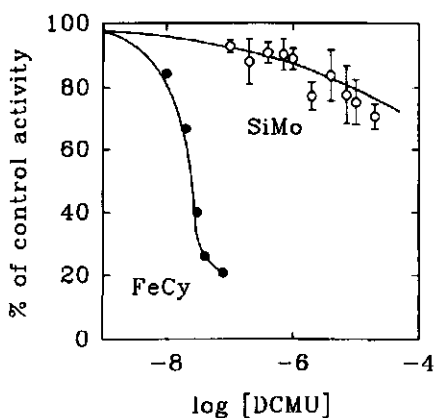
Fig. 4.3. DCMU titration curves. (open circles) SiMo as electron acceptor; (filled circles) FeCy as electron acceptor. Initial rate of oxygen evolution expressed in % of the control rate was measured at pH 7.6.

DCMU does not inhibit more than 30% of the SiMo-mediated electron transport activity. However, using probit analysis (Finney 1952) the I_{50}-value for DCMU in the presence of SiMo can be estimated to be 800 μM (Table 4.1).

Ioxynil is a much more effective inhibitor of SiMo reduction than DCMU (Table 4.1). In Fig. 4.4 the ioxynil titration curves with SiMo and FeCy as electron acceptor are plotted. In the presence of SiMo, inhibition of electron transport by ioxynil is biphasic. Pallett and Dodge (1978) also found a biphasic titration curve of the Hill

Table 4.1. The effect of the herbicides DCMU, ioxynil and dinoseb on the Hill reactions with FeCy and SiMo

Herbicide	I_{50} FeCy (nM)	I_{50} SiMo $(\mu M)^a$	ratio
DCMU	32	800	25000
ioxynil[b]	130[c]	52-74[d]	400-570
dinoseb[e]	2800	21	8

a In all cases SiMo was added 2 min before the start of the measurement.

b Ioxynil was dark incubated with the thylakoids for 15 min.

c This value differs from the one in Schansker and Van Rensen (1992) because the dark incubation time with ioxynil was 15 min instead of 5 min.

d This value differs from the value in Schansker and Van Rensen (1992) because the titration curve was reinterpreted (see Fig. 4.4). A range of values is given because between 15 and 20% accessibility of the donor side to ioxynil little change in the fit was found.

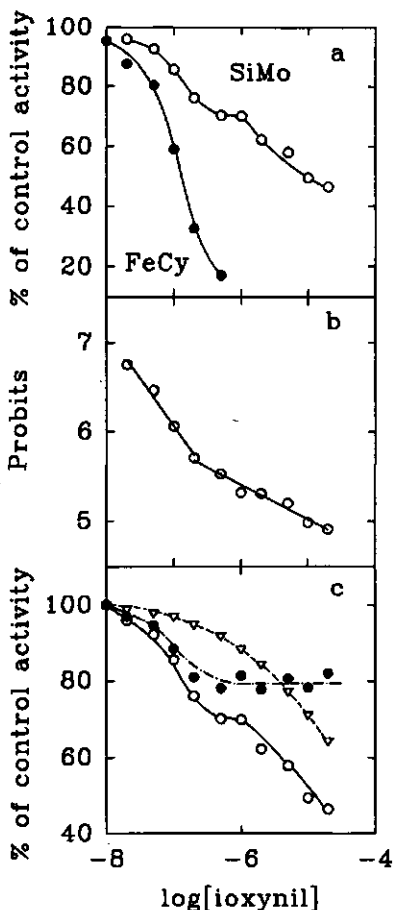e Dinoseb was dark incubated with the thylakoids for 5 min.

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Fig. 4.4. Ioxynil titration curves. a. Comparison of the inhibition of SiMo-mediated (open circles) and FeCy-mediated electron transport (filled circles). The initial rate of oxygen evolution expressed as % of the control rate was measured at pH 7.6. b. Probit analysis of the inhibition of the SiMo-mediated electron transport. c. Deconvolution of the ioxynil titration curve using SiMo as electron acceptor. High affinity component (filled circles); low affinity component (triangles) and total curve (open circles). The curve was deconvoluted assuming a. two binding sites for ioxynil (Fig. 4.4b) one on the donor side and one on the acceptor side, and b. only part of the donor side binding sites were accessible to ioxynil (Pfister and Schreiber 1984). The accessibility of the binding sites on the donor side was varied. Two criteria were used to assess the fit: a. the sum of the sigmoid had to equal the original curve, and b. the two sigmoids should produce straight probit lines. A good fit was found assuming 15 to 20% accessibility of the donor side binding sites (Fig. 4.4c).

reaction with ioxynil using SiMo as an electron acceptor. This can be explained by assuming a second ioxynil inhibition site at the donor side of PS II (= lumen side of the thylakoid membrane) (Oettmeier *et al.* 1987b).

Dinoseb is the best inhibitor of SiMo-mediated electron transport (Table 4.1); it did not show a biphasic behaviour.

Lineweaver Burk plots
Another way to analyze the interaction between SiMo and an inhibitor is to make a SiMo titration curve and to plot it as a Lineweaver Burk plot. This is done for DCMU. At pH 7.6 the interaction between SiMo and DCMU is apparently competitive (Fig. 4.5).

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However, increasing the DCMU-concentration from 1 μM to 10 μM does not increase the K_m with a factor 10. A ten times higher concentration increases the K_m only from ~40 μM SiMo to ~70 μM SiMo. This suggests that the binding sites of SiMo and DCMU are not identical, although only one species can be bound to the thylakoid membrane at the same time. If, however, the same experiment is repeated at pH 6.3, the results are totally different. At pH 6.3 the interaction between SiMo and DCMU is non-competitive.
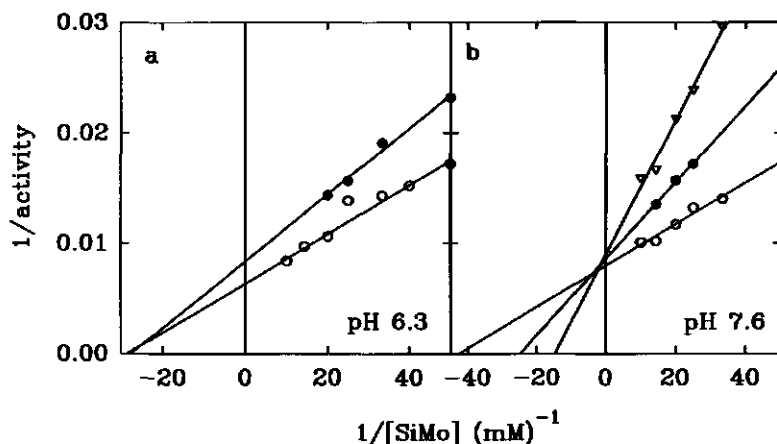

Fig. 4.5. Effect of the pH on the relation between SiMo and DCMU. Lineweaver Burk plots of the effect of DCMU on the SiMo titration curve at pH 6.3 (a) and at pH 7.6 (b). (open circles) -DCMU; (filled circles) +1 μM DCMU; (triangles) +10 μM DCMU. SiMo was incubated for 2 min in the reaction medium to which 1 μM DBMIB was added before the light was turned on. Activity is the initial rate of oxygen evolution expressed in $\mu mol(O_2).mg^{-1}(Chl).h^{-1}$.

SiMo titration curves: addition in light or after dark incubation
In Fig. 4.6 the effect of 2 min dark incubation with SiMo (pH 7.6) is compared with addition of SiMo in the light. Two features should be noticed. In the first place there is a large difference in the maximum electron transport activity (V_{max}). A second feature is the difference in K_m. A two minutes dark incubation with SiMo induces a decrease of the K_m compared with adding SiMo in the light.

In Table 4.2 K_m and V_{max} values from both pH 7.6 (discussed above) and pH 6.3 are compared. The differences in K_m nor the differences in V_{max} occur at pH 6.3.
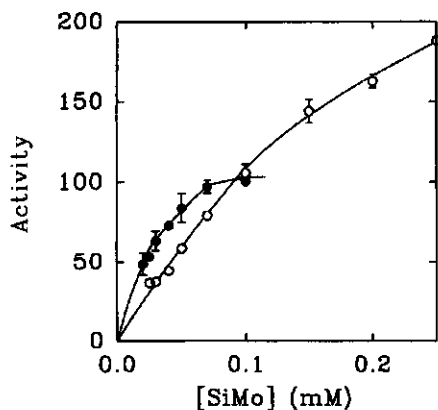
Fig. 4.6. Effect of the dark incubation time with SiMo on the SiMo titration curve. (open circles) SiMo added in the light; (filled circles) two minutes dark incubation with SiMo. The initial rate of oxygen evolution expressed in $\mu mol(O_2)$. $mg^{-1}(Chl).h^{-1}$ was measured in the presence of 1 μM DBMIB at pH 7.6.

Table 4.2. K_m and V_{max}-values of SiMo-reduction under different experimental conditions in the presence of 1 μM DBMIB.

pH	dark incubation time with SiMo (min)	V_{max} ($\mu mol(O_2).mg^{-1}(Chl).h^{-1}$)	K_m (μM)
6.3	0	155	22
	2	154	35
7.6	0	454	343
	2	126	24

Role of bicarbonate

Since Stemler (1977) reported that SiMo can displace bicarbonate in a time dependent way, we studied the role of bicarbonate in the SiMo-mediated Hill reaction. In Fig. 4.7 the interaction between bicarbonate and SiMo is plotted as a Lineweaver Burk plot. We had expected a competitive interaction, it appears to be non-competitive. Since this would mean that in the end SiMo would always displace all bicarbonate, the equilibrium measurements were unnecessary. Instead, the SiMo-mediated electron transport in the presence or absence of 1 μM DBMIB and in the presence of 0, 10 or 20 mM bicarbonate was followed in time (Fig. 4.8a,b,c). In the absence of DBMIB, SiMo accepts electrons at both PS I and PS II. Displacement of bicarbonate by SiMo is expected to abolish the
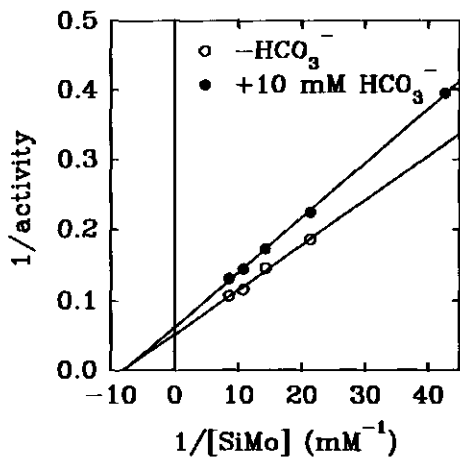
Fig. 4.7. Effect of the addition of bicarbonate on the SiMo titration curve. (open circles) -bicarbonate; (filled circles) +10 mM bicarbonate. The initial rate of oxygen evolution expressed in $\mu mol(O_2).mg^{-1}(Chl).h^{-1}$ was measured in the presence of 1 μM DBMIB at pH 7.6.

electron transport to PS I. Addition of bicarbonate slows the decrease of activity although it does not prevent it (Fig. 4.8a). After about two minutes in all three experiments the same level of activity was reached. In Fig. 4.8b the same experiment was done but 1 μM DBMIB was added to block electron transport to PS I. In Fig. 4.8b only PS II activity was measured. The interesting feature of Fig. 4.8b is a transient increase of SiMo mediated electron transport by 15% in the presence of bicarbonate. In Fig. 4.8c the difference between Figs. 8a and 8b is plotted. This plot illustrates that the slower decline of activity in the presence of bicarbonate (Fig. 4.8a) can be explained by the increase of PS II activity. We did not observe a clear inhibitory effect of bicarbonate as was reported by others (Barr and Crane 1976, Stemler and Jursinic 1983, Stemler and Murphy 1983). However, those authors did their experiments in the presence of DCMU, which may account for the apparently contradictory results. There was no transient increase of SiMo-mediated electron transport in the absence of DBMIB. As a possible explanation for the transient increase of SiMo-mediated electron transport in the presence of 1 μM DBMIB we suggest that bicarbonate may have made Q_B non-reducing centers accessible to SiMo. In Fig. 4.8b an initial inhibition of SiMo-mediated electron transport by the addition of bicarbonate can be seen. This is in agreement with the assumption that SiMo has to displace bicarbonate before it can accept electrons.

Triazine resistance

We have also investigated the effect of a change of serine at position 264 of the D1 protein into glycine on the binding of SiMo by comparing thylakoids from triazine resistant and triazine sensitive plants of Chenopodium album. This was done by making light saturation curves and SiMo titration curves in the presence of 1 μM DBMIB. The light saturation curves indicated that the mutation had no effect on the maximum activity. Using 0.1 mM SiMo in both cases a V_{max} of 149 $\mu mol(O_2).mg^{-1}(Chl).h^{-1}$ was found. This
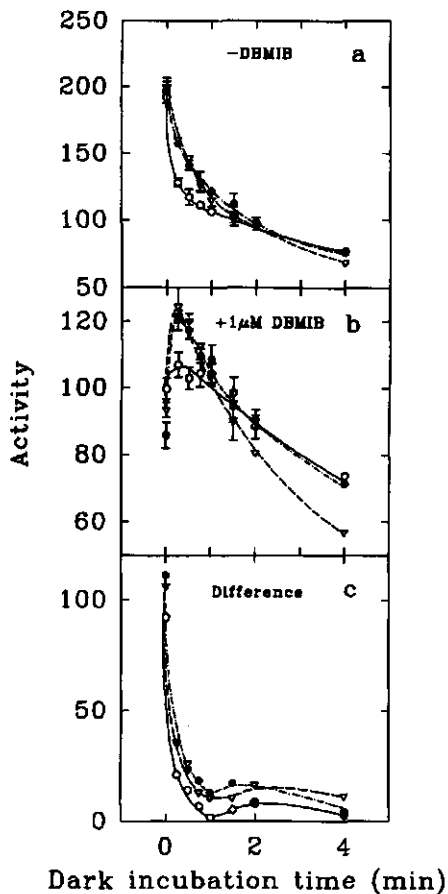
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Fig. 4.8. SiMo-mediated electron transport measured as a function of the dark incubation time with SiMo. a. -DBMIB; b. +1 µM DBMIB; c. the difference between a and b. (open circles) no bicarbonate added; (filled circles) 10 mM bicarbonate; (triangles) 20 mM bicarbonate. SiMo (0.1 mM) was added to the pea thylakoids at time 0 and after the indicated dark incubation times, the light was turned on and the initial rate of oxygen evolution expressed in $\mu mol(O_2).mg^{-1}(Chl).h^{-1}$ was measured at pH 7.6.

confirms the work of Jansen *et al.* (1986) who reported similar activities of the SiMo-mediated Hill reaction at light saturation of resistant and sensitive chloroplasts of *C. album*. The mutation did however have an effect on the K_m-value: 860 $\mu mol.m^{-2}.s^{-1}$ for the triazine-sensitive thylakoids and 1430 $\mu mol.m^{-2}.s^{-1}$ for triazine-resistance thylakoids (Fig. 4.9). As expected, SiMo titration curves at high irradiance (5700 $\mu mol.m^{-2}.s^{-1}$) gave almost the same results for both the triazine-resistant and the triazine-sensitive thylakoids (not shown). Using a low irradiance (510 $\mu mol.m^{-2}.s^{-1}$), a more pronounced difference was found. Again there was no effect on the V_{max}, which was in both cases about 180 $\mu mol(O_2).mg^{-1}(Chl).h^{-1}$, but the K_m was 0.18 mM for the triazine-sensitive thylakoids and 0.29 mM for the triazine-resistant thylakoids (not shown). In summary triazine-resistant thylakoids have a lower affinity for SiMo, but this effect can be diminished by the use of high irradiances.

Discussion

There are several questions pertinent to the use of SiMo as a probe. These questions are: (1) Where and how does SiMo bind? (2) What is the nature of the interaction of SiMo with PS II inhibitors and bicarbonate? And (3) Where does SiMo get its electrons from?

The key to the understanding of the interaction between thylakoid membranes and
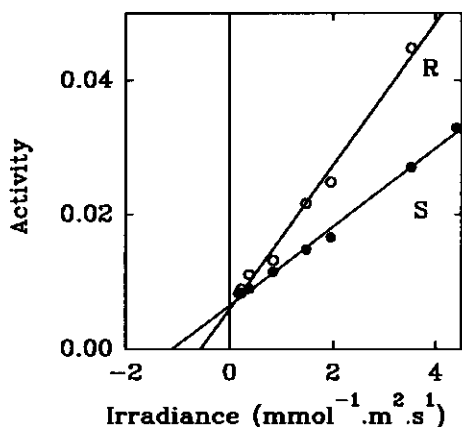
Fig. 4.9. Comparison of the irradiance response curves of atrazine-sensitive and atrazine-resistant thylakoids of Cheno- podium album. *The irradiance and the initial rate of oxygen evolution expressed in* $\mu mol(O_2).mg^{-1}(Chl).h^{-1}$ *were plotted double reciprocally. SiMo (0.1 mM) was added directly after the light was turned on.*

SiMo is its displacement of bicarbonate as observed by Stemler (1977). The displacement of bicarbonate by SiMo causes time-dependent changes in activity and pH-dependent changes in the interaction with inhibitors.

The binding sites for SiMo
In the past up to three binding sites for SiMo have been reported: two binding sites at photosystem II (Giaquinta *et al.* 1975, Berg and Izawa 1977) and one at PS I. There is general agreement about one site at PS I and one site at PS II. The PS I site is KCN sensitive (Giaquinta *et al.* 1975, Berg and Izawa 1977, Böger 1982). There is also a general agreement about at least one binding site at PS II. A third binding site, also located at or close to PS II is debatable. Böger (1982) and Fig. 4.7 (in this paper) show that if thylakoids are inhibited by KCN and DBMIB, respectively, the relation between the SiMo concentration and the oxygen-production can be described by Michaelis Menten kinetics (the Lineweaver Burk plots give straight lines). This strongly suggests that there exists only one binding site before the KCN/DBMIB block. A second strong argument in favour of only two binding sites for SiMo (one at PS I and one at PS II) is that experiments suggesting the existence of a third binding site can easily be explained in a different way. Berg and Izawa (1977) made a DCMU titration curve using KCN inhibited thylakoids and SiMo as electron acceptor. They found a DCMU sensitive and a DCMU insensitive component in their curve. However they added the electron acceptor directly after turning on the light. This means that they did not measure under equilibrium conditions. As SiMo needs time to displace all the DCMU (Berg and Izawa 1977) part of the PS II complexes had still bound DCMU and these PS II centers were still unable to donate electrons to SiMo. In this way also SiMo sensitive and insensitive centers are created. Giaquinta *et al.* (1975) used also KCN treated thylakoids. They studied the

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interaction between the SiMo concentration and electron transport activity. They found that increasing the SiMo concentration did not lead to saturation of the electron transport activity. However, this observation can also be explained by assuming leakage of electrons past the KCN block. This phenomenon was also seen at high irradiance as a irradiance saturation curve was made with SiMo as electron acceptor and 1 μM DBMIB to prevent electron transport to PS I (Schansker and Van Rensen 1992).

Interaction between SiMo and bicarbonate

Stemler (1977) observed that bicarbonate was displaced by SiMo in a time dependent way. We have characterized this displacement in this paper (Figs. 7 and 8). When SiMo was added after turning on the light (non-equilibrium conditions), Lineweaver Burk plots suggested that the interaction between SiMo and bicarbonate was non-competitive (Fig. 4.7). Both Stemler (1979) and Diner and Petrouleas (1990) proposed that bicarbonate is imprisoned by PS II. Stemler (1979) observed that bicarbonate was only lost from PS II as the redox state of Q_B changed. Diner and Petrouleas (1990) proposed that bicarbonate could associate and dissociate many times from the non-heme iron before it was lost to the medium. One assumption that can be made is that as long as a bicarbonate molecule is bound to the non-heme iron SiMo cannot displace it. The moment bicarbonate dissociates from the non-heme iron it looses its competitive power. SiMo is then able to break the bicarbonate "prison" open and in the process bicarbonate is lost to the medium. Discussing the binding of phenol type herbicides, Trebst (1987) proposed an induced fit, meaning that the inhibitor pushes away amino acid residues in its approach to its binding site. This may also happen in the case of the binding of SiMo. Once SiMo is bound to PS II, it may block the rebinding of bicarbonate. This process is the same as Diner and Petrouleas (1990) have proposed for the interaction between bicarbonate and NO. A consequence of this mechanism would be that addition of bicarbonate has no effect on bicarbonate binding under equilibrium conditions. The data presented in Fig. 4.8 are in agreement with this implication. After two minutes of dark incubation of the thylakoids with SiMo, there is no longer an effect of the addition of bicarbonate on SiMo-mediated electron transport.

Effect of triazine resistance on SiMo binding

In triazine resistant plants Ser264 of the D1 protein is altered into glycine. Khanna *et al.* (1981) observed that this change resulted in a 2-fold lower affinity of PS II for bicarbonate. Because of the above mentioned interaction between bicarbonate and SiMo an increase of the accessibility of PS II to SiMo in triazine resistant plants was expected. However the data presented in Fig. 4.9 indicate that PS II reaction centers of triazine resistant plants are 1.67 times less accessible to SiMo than those of triazine sensitive plants. Obviously Ser264 is more important to SiMo binding than a lower affinity of PS II for bicarbonate. The difference between triazine resistant and triazine sensitive thylakoids

is diminished by increasing the light irradiance and is almost non existant at 5700 μmol.m^{-2}.s^{-1} irradiance (not shown).

*Interaction of SiMo with PSII inhibitors*

Some details of the interaction of the individual herbicides with SiMo have already been mentioned under the Results. As can be seen in Table 4.1, DCMU, ioxynil and dinoseb differ strongly in their effects on SiMo binding. DCMU is a poor inhibitor of SiMo binding and dinoseb is a very good inhibitor. To interpret these results, we use the binding model proposed by Trebst (1987). Trebst suggested that there exist two families of inhibitors binding to the Q_B binding niche. In the histidine family, to which dinoseb belongs, herbicides bind close to His215. This histidine is also a ligand to the non-heme iron. In the serine family, to which DCMU belongs, herbicides bind close to Ser264. The latter binding site is much more accessible to inhibitors than the first. There are several observations indicating that ioxynil (and also bromoxynil) do not behave in the same way as other members of the histidine family (e.g. Vermaas *et al.* 1984) which is supported by the intermediate position of ioxynil in Table 4.1. Also of importance for the understanding of the interaction of SiMo with PS II inhibitors is the interaction between CO_2 depletion and herbicide binding affinity. In CO_2-depleted thylakoids the affinity for atrazine is 3.9 times lower, there is little effect on the affinity for ioxynil and the affinity for i-dinoseb is 3.5 times higher (Khanna *et al.* 1981, Vermaas *et al.* 1982, Vermaas 1984). Expecting DCMU to behave in the same way as atrazine, we find the same hierarchy again. Knowing that SiMo also interacts with bicarbonate indicates that the binding of bicarbonate plays a key role in the interaction between PS II inhibitors, SiMo and bicarbonate. The differences in Table 4.1 can now be explained by differences in the orientation of the herbicides within the Q_B binding niche, differences in the effects of bicarbonate on the binding affinity of herbicides or a combination of both effects.

A closer look at the interaction between DCMU and SiMo (Fig. 4.5) illustrates that this interaction is pH-dependent. At pH 7.6 the interaction is competitive; at pH 6.3 the interaction is non-competitive. The difference may be due to the chemical characteristics of CO_2. CO_2 dissolves about 10.5-fold better in water of pH 7.6 than in water of pH 6.3. Additionally about 45% of the dissolved CO_2 is bicarbonate at pH 6.3 compared with 95% at pH 7.6 (Nobel 1974). The combined effect is that at pH 6.3, 22 times less bicarbonate is present compared to pH 7.6. Bicarbonate is needed for high affinity of PS II for DCMU. This means that DCMU can only compete with SiMo if bicarbonate is able to rebind. At pH 7.6 the bicarbonate concentration is high enough to achieve rebinding in the presenced of DCMU; at pH 6.3 this is not the case. The observation that at pH 7.6 the interaction between DCMU and SiMo is competitive also indicates that PS II has a much lower affinity for reduced SiMo. This effect was also observed by Graan (1986). The data in Fig. 4.5 explain why Graan (1986) at pH 6.5 found a non-competitive interaction between SiMo and DCMU and Böger (1982) found a competitive interaction at

pH 8.0. Figure 5 can also be used to explain the observation of two pH sensitive binding sites for SiMo at PS II by Barr and Crane (1980).

Localization of the SiMo binding site
The binding site for SiMo in the PS II region has been a matter of debate. Graan (1986) proposed that SiMo occupied the Q_B-binding site. Since quinones are better electron acceptors than SiMo and also bind to the Q_B binding site, Graan concluded that SiMo was a "superfluous" electron acceptor, *i.e.*, of not much use to probe PS II. There are however several arguments against this conclusion. (1) SiMo is an extremely large and quite hydrophilic compound making the hydrophobic Q_B-binding site less accessible to SiMo. (2) More importantly, there is no correlation between the binding affinities of DCMU, ioxynil and dinoseb and their ability to inhibit the SiMo-mediated electron transport (Table 4.1). (3) The interaction between SiMo and DCMU is not really a competitive one (increasing the DCMU-concentration ten times does not increase the K_m-value for SiMo ten times). (4) SiMo displaces bicarbonate. All these arguments can be accounted for if one assumes that SiMo creates a binding niche between both parallel helices of the D1 and D2 protein (see Fig. 4.10). We suggest, that the non-heme iron donates the electrons to SiMo.
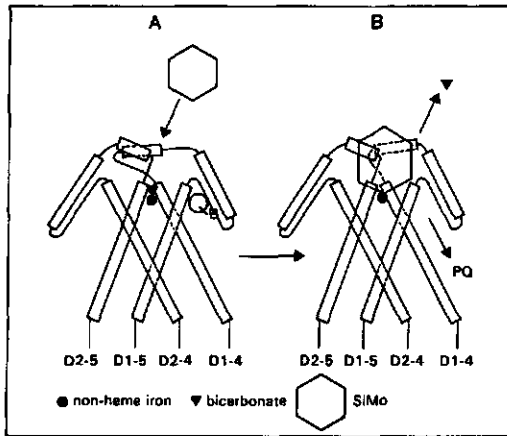
Fig. 4.10. A self-explanatory scheme of the suggested binding site of SiMo. D2-5 stands for the 5th helix of the D2 protein; others have a similar meaning.

Relation of bicarbonate with photoinhibition
It was demonstrated in Fig. 4.7 that the interaction between bicarbonate was non-competitive. Comparable results were reported by Diner and Petrouleas (1990) who

studied the interaction between NO and bicarbonate (see above). In Chapter 3 it was postulated that the release of bicarbonate is the cause of photoinhibition. The observed non-competitive interaction between bicarbonate and SiMo, respectively NO, means that addition of bicarbonate (Sundby 1990) is only expected to give a transient protection against photoinhibition. To prove that bicarbonate release is the cause of photoinhibition other experiments have to be devised.

5 Mechanism of photoinhibition in pea thylakoids: effects of irradiance level and pH

Abstract

Photoinactivation primarily affects the acceptor side of PSII. The donor side of PSII is also sensitive to photoinhibition, but, at pH 7.6, the inactivation rate of the donor side is almost 4 times slower. Inactivation of both donor and acceptor side are light dose dependent. The results indicate that donor and acceptor side inactivation are independent processes. The presence of DCMU during the photoinhibitory treatment does not affect the rate of donor side inactivation. Donor and acceptor side inactivation are pH dependent. The pH dependence of both processes follows separate patterns. The pH dependence of acceptor side inactivation could be explained by assuming that light causes the release of bicarbonate from PSII. Further, photoinhibition seems to affect the accessibility of PSII to SiMo indicating a conformational change. A photoinhibitory treatment eliminates photosynthetic control and thus affects the proton permeability of thylakoid membranes. Lowering the pH from 7.6 to 6.4 accelerates the elimination of photosynthetic control twofold. A photoinhibitory treatment starts to increase the proton permeability of the thylakoid membrane after about 70% of PSII is inactivated. Possibly, radicals formed by inactivated PSII reaction centers causing lipid peroxidation are responsible for this effect.

Introduction

The photosynthetic apparatus not only utilizes light but it can also be damaged by light. The process of inactivation of photosynthesis by light is called photoinhibition. Photoinhibition is defined here as the inactivation of photosynthetic electron transport by light. Much research has been directed towards the elucidation of the mechanism by which light inhibits PSII. The double reduction of Q_A was postulated to be the cause for inhibition (Van Mieghem *et al.* 1989). Though double reduction of Q_A has been observed to occur under anaerobic conditions (Van Mieghem *et al.* 1989, Andersson *et al.* 1989,

Styring *et al.* 1990, Van Wijk *et al.* 1992, Vass *et al.* 1992), little experimental proof for the involvement of this process in photoinhibition has been obtained under aerobic conditions (see Chapter 8 for a discussion). Other hypotheses, describing the primary cause of photoinhibition in mechanistic terms, are so far insufficiently backed by experimental proof. Sundby (1990, 1992) tried to give bicarbonate a functional role in this process, by studying the protective effect of added bicarbonate during a photoinhibitory treatment. Experiments with nitric oxide (NO) (Diner and Petrouleas 1987) and silicomolybdate (SiMo) (Chapter 4) have shown that bicarbonate is displaced non-competitively. This implies that the addition of bicarbonate is not a very effective way to prevent the release of bicarbonate. Here, we have designed other experiments to study if bicarbonate plays a role in the mechanism of inactivation.

The results in Chapter 4 have shown that SiMo can be used as a probe for the acceptor side of PSII. Further it was found that SiMo-reduction at saturating irradiance was relatively insensitive to the presence of DCMU and bicarbonate. Utilizing the differential effects of light and pH on the inhibition of SiMo- and FeCy-mediated electron transport, the mechanism by which light inhibits PSII was studied. The thylakoid pH gradient (ΔpH) is thought to protect the photosynthetic apparatus from photoinhibition. In the last part of this study the relationship between changes in the permeability of the thylakoid membrane for protons and the inactivation kinetics of PSII were studied.

Materials and methods
Plant growth conditions, isolation of thylakoids, reaction mixtures, preparation of SiMo and use of SiMo were as described in Chapter 4.

DCPIPH$_2$/ascorbate→methylviologen reaction
Electron transport activity from DCPIPH$_2$/ascorbate to MV was assayed in the presence of the following additions: 40 μM DCPIP, 2 mM ascorbate, 20 μM MV, 100 units SOD to accelerate the formation of H_2O_2, 2 mM DTE to prevent the formation of O_2 out of H_2O_2. Thylakoids were uncoupled with 5 μM gramicidin and the buffers were 50 mM MES at pH 6.4 and 50 mM Tricine at pH 7.6. The measuring irradiance was 2200 μmol.m^{-2}.s^{-1}.

Photoinhibition experiments
Thylakoid membranes (25 μg/ml) and reaction medium were added to the reaction vessel of a Gilson oxygraph and irradiated with 'white light' of about 5700 μmol.m^{-2}.s^{-1} provided by a slide projector. The projector was equiped with a water filter and the reaction vessel was cooled by water. For further details see the figure legends.

Determination of K$_m$, V$_{max}$ and apparent quantum yield
These parameters were derived from SiMo titration curves (K$_m$ and V$_{max}$) and irradiance

response curves (K_m, V_{max} and apparent quantum yield). Both types of curves were plotted as Lineweaver-Burk plots. $1/K_m$ was calculated as the intersection of the regression line with the x-axis of the Lineweaver-Burk plot. The apparent quantum yield was calculated as the reciprocal slope of the regression line of the Lineweaver-Burk plot.

Results

Primary site of photoinhibitory damage

In Fig. 5.1, SiMo was used to study the primary site of photoinhibitory damage. As was demonstrated in the previous Chapter there are two sites in the electron transport chain where SiMo accepts its electrons. The first site is located close to the non-heme iron between Q_A and Q_B (Fig. 4.2). The second site is located beyond the cyt b_6/f-complex, probably on the acceptor side of PSI. Both sides are mutually exclusive. If SiMo is added immediately after the light is turned on a mix of whole chain and PSII-mediated electron transport is measured (Fig. 5.1a and Fig. 4.2). Electron transport to the second acceptor
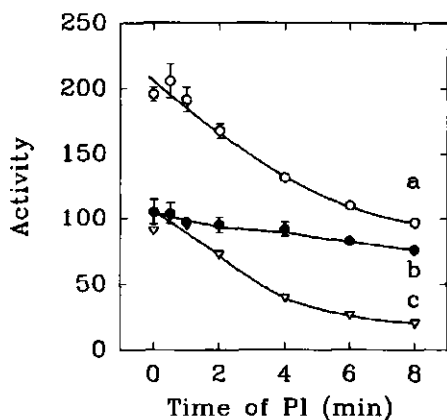

Fig. 5.1. Electron transport activity from H_2O to SiMo as a function of the time of the photoinhibitory treatment (PI): a. measured in the absence of DBMIB (open circles), b. in the presence of 1 μM DBMIB (filled circles) and c. the difference between a and b (open triangles). Electron transport activity is expressed in $\mu mol(O_2).mg^{-2}(Chl).h^{-1}$. DBMIB and NH_4Cl (5 mM) were added after the photoinhibitory treatment; SiMo (0.1 mM) was added at the moment the actinic light was turned on. Bars represent standard deviation (n = 3-4)

site is prevented by the addition of 1 μM DBMIB (Fig. 5.1b). Whole chain electron transport was determined by subtracting the rate of PSII-mediated electron transport from the mixed rate (Fig. 5.1c). This was done under the assumption that two separate populations of electron transport chains were measured. Without photoinhibition (PI = 0 min) the activity of curves b and c are the same. This indicates that under these conditions 50% of the PSII reaction centers bind SiMo on addition and the other 50% of the reaction centers are still able to transfer electrons to the plastoquinone pool. Whole chain electron transport was affected much more by the photoinhibitory treatment than

electron transport to Q_A (the kinetics of this inactivation are comparable to that using FeCy as electron acceptor). As electron transport between cyt b_6/f and the acceptor side of PSI is less affected by the photoinhibitory treatment under our conditions (see later, e.g., Fig. 5.7), these data indicate that the primary site of photoinhibition is to be found between Q_A and the plastoquinone pool. Electron transport to Q_A as measured by SiMo is also sensitive to photoinhibition indicating that the donor side of PSII is sensitive to a photoinhibitory treatment though much less than electron transport between Q_A and the plastoquinone pool.

Irradiance dependence of the treatment
The irradiance dependence of two partial reactions was determined: $H_2O \rightarrow$ SiMo and $H_2O \rightarrow$ FeCy. If the inactivation of these two reactions is determined by the dose, the irradiance level multiplied by the halftime of inactivation ($I \times t_{1/2}$) should be constant. As illustrated in Fig. 5.2 this is true for both the reaction from $H_2O \rightarrow$ SiMo and the reaction from $H_2O \rightarrow$ FeCy. Only at the lowest inactivation irradiance used a deviation from linearity was found for the inactivation of the $H_2O \rightarrow$ SiMo reaction but this is probably caused by a contribution of dark inactivation of the thylakoids due to the long incubation times.
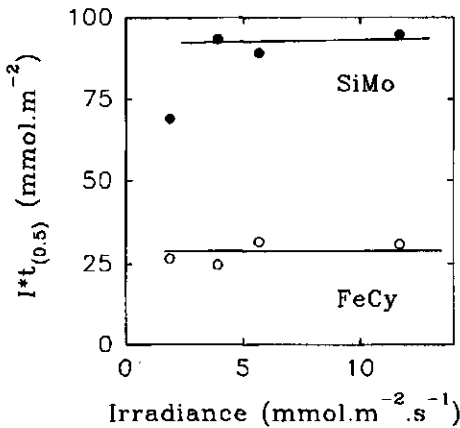
Fig. 5.2. Effect of photoinhibitory irradiance level on the light dose needed to inactivate the reactions $H_2O \rightarrow$ SiMo (filled circles) and $H_2O \rightarrow$ FeCy (open circles) by 50%. At every irradiance level the halftime of inactivation was determined from plots of electron transport activity versus photoinactivation time. The pH of the medium was 7.6.

Repetition of the experiment of Fig. 5.2 at pH 6.7 indicated that lowering the pH had little effect on the relation between irradiance level and light dose in the case of inactivation of SiMo-mediated electron transport (not shown). In the case of FeCy-mediated electron transport, a 20% decrease of the dose needed to inactivate 50% of the electron transport activity was observed. In both cases the relation between the light dose and the halftime of inactivation, indicating a dose response dependence, was also found at pH 6.7.

The existence of a dose response relationship for the range of irradiances tested

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indicates that the use of a high inactivation irradiance (6 mmol.m^{-2}.s^{-1}) does not lead to deviating results.

pH-profile of inactivation

As explained in Chapter 3, the working hypothesis of this thesis is that the inactivation of the acceptor side of PSII is brought about by the irreversible loss of bicarbonate from its binding site. It was shown that exchange of labeled bicarbonate with the medium is virtually non-existent above pH 7 and becomes progressively facilitated as the pH drops (Stemler 1979, Stemler and Murphy 1983, Diner and Petrouleas 1990). As their experiments were carried out in the dark it is possible that exchange would have occurred at more alkaline pH values in the light. Stemlers observation (1979) indicates that lowering the pH will facilitate release of bicarbonate. If release of bicarbonate is the cause of photoinhibition it is expected that lowering the pH below 7 will increase the rate of inactivation of FeCy-mediated electron transport while SiMo-mediated electron transport will remain unaffected. In Fig. 5.3 the results of this experiment are presented.
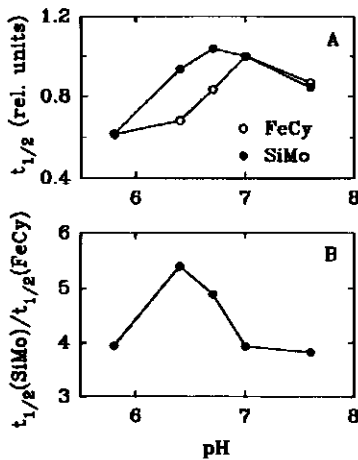
Fig. 5.3. pH dependence of the halftime of photoinactivation $(t_{1/2})$ of the reactions $H_2O \rightarrow SiMo$ (filled circles) and $H_2O \rightarrow FeCy$ (open circles). In Fig. 5.3a the inactivation halftimes of both reactions were normalized to the value at pH 7.0. In Fig. 5.3b the ratios between the halftime of inactivation of $H_2O \rightarrow SiMo$ and the halftime of inactivation of $H_2O \rightarrow FeCy$ are plotted. Halftimes of inactivation were determined as in Fig. 5.2. The buffers used were MES pH 5.8-6.7, Hepes pH 7.0-7.3 and Tricine for the highest pH values.

For the inactivation of SiMo-mediated electron transport a broad optimum peaking around pH 6.7 is found. The pH-profile of inactivation follows more or less the pH-profile of the photosynthetic activity of PSII (Gould and Izawa 1973b, Izawa 1980). The relationship between the inactivation of FeCy-mediated electron transport and the pH follows a much more complicated pattern. As hypothesized the rate of inactivation increases sharply below pH 7. The ratio between the inactivation halftimes of SiMo- and FeCy-mediated electron transport is maximal around pH 6.4 (Fig. 5.3b). It could be argued that the pH-profile of the inactivation of the FeCy-mediated electron transport reaction follows the pH-profile of whole chain electron transport activity. However, no relationship between

the pH-profile of FeCy-mediated electron transport and the pH-profile of the halftime of inactivation of FeCy-mediated electron transport was found (not shown, see also Gould and Izawa 1973b).

Residual activity

It is often very difficult to inactivate the last 20% of control electron transport activity. At pH 7.6 we also observed this effect (Fig. 5.4). In this figure the inactivation curves measured using FeCy as electron acceptor at different pH values are plotted. The data in Fig. 5.4 indicate that below pH 6.7 this phenomenon is no longer observed.
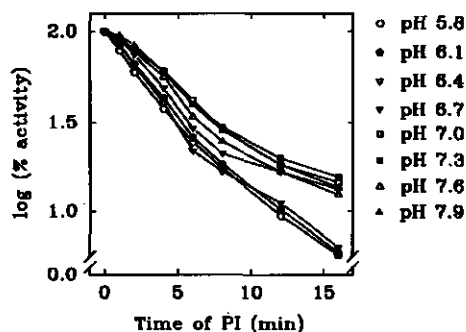

Fig. 5.4. pH dependence of the relationship between the duration of the photoinhibitory treatment (time of PI) and the relative electron transport activity of $H_2O \rightarrow FeCy$ plotted on a log scale. If the duration of the photoinhibitory treatment was longer than 8 min a deviation from the exponential decline of activity is seen at pH 6.7 and above.

Irradiance response curves

A photoinhibitory treatment can have several effects on the kinetics of ongoing reactions. In the case of SiMo-mediated electron transport, the binding site for SiMo can be affected, as well as the number of binding sites available (related to the maximum electron transport rate) and the intrinsic quantum yield of PSII for electron transfer. These effects are studied by determining irradiance response curves of control and photoinhibited thylakoids at pH 6.4 and pH 7.6 after a photoinhibitory treatment of 8 min. (The duration of this treatment is long enough to inactivate 70% or more of whole chain electron transport, Fig. 5.4). Double reciprocal curves at pH 7.6 indicate that after a photoinhibitory treatment of 8 min the maximum electron transport rate (V_{max}) had declined 1.4-fold while the photon flux density yielding a half maximal rate of electron flow (K_m) had increased 5.8-fold and the apparent quantum yield had declined 7.5-fold (Fig. 5.5). The data in Fig. 5.5 indicate that the loss of activity at limiting photon flux densities is larger than the loss of activity at saturating photon flux densities. At pH 6.4 the V_{max} had declined 1.2 fold, the K_m is increased 3.8 fold and the quantum yield decreased 4.5 fold (not shown). These data indicate that the effects at pH 7.6 and 6.4 are comparable.
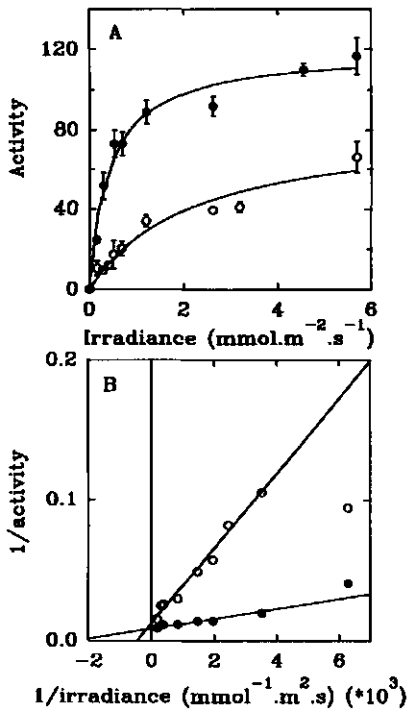
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Fig. 5.5. Effect of 8 min photoinhibitory treatment on the irradiance response curve at pH 7.6 with SiMo as electron acceptor. Activity ($\mu mol(O_2).mg^{-1}(Chl).h^{-1}$) was measured after 1 min darkness following the completion of the photoinhibitory treatment. SiMo (0.1 mM) was added in the light. The measurement irradiance was varied with neutral density filters. (A) irradiance response curves; (B) double reciprocal plots of the irradiance saturation curves. (filled circles) no photoinhibitory treatment; (open circles) 8 min photoinhibitory treatment. No photoinhibitory treatment: V_{max} = 119 $\mu mol(O_2).mg^{-1}(Chl).h^{-1}$, K_m = 415.9 $\mu mol.m^{-2}.s^{-1}$ and the apparent quantum yield = 0.286; 8 min photoinhibitory treatment: V_{max} = 84.2 $\mu mol(O_2).mg^{-1}(Chl).h^{-1}$, K_m = 2200 $\mu mol.m^{-2}.s^{-1}$ and the apparent quantum yield = 0.038.

Protective effect of DCMU

In Fig. 5.6 the effect of DCMU on the inactivation of SiMo-mediated electron transport is shown. SiMo was incubated for 2 min with the treated thylakoids on the assumption that the presence of DCMU will prevent the accumulation of reduced components. In this case it is difficult to perform a control experiment. Addition of DCMU after the photoinhibitory treatment would mean that during the photoinhibitory treatment reducing equivalents can have accumulated which would reduce SiMo during the 2 min incubation period (Schansker and Van Rensen 1992). As a substitute the data in Fig. 5.1a and data in Fig. 5.6 were compared which indicate that the addition of DCMU has no effect on the inactivation of SiMo-mediated electron transport.

Photoinhibition and proton permeability

To study the effect of photoinhibition on membrane permeability it is necessary to find a partial electron transport reaction that is only slightly affected by a photoinhibitory treatment. A decline of electron transport activity makes it *a priori* difficult to separate the effect of a decrease in the built up of the transmembrane pH gradient and leakage of

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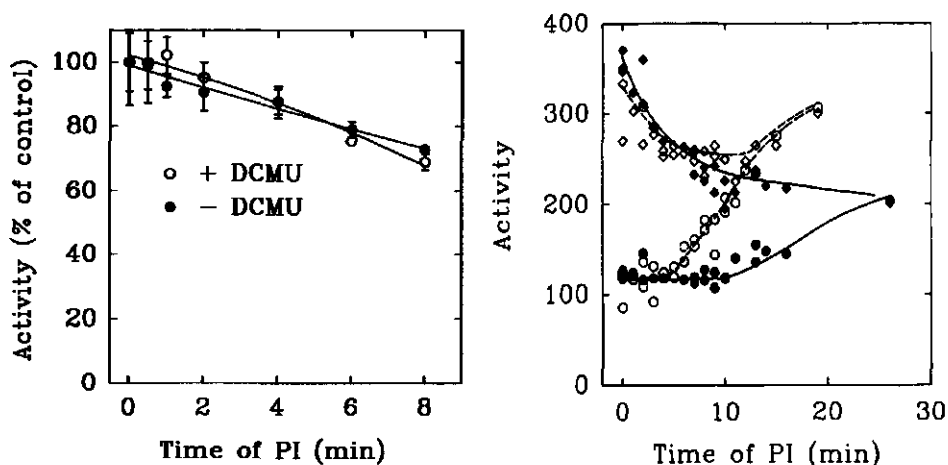
Fig. 5.6. Effect of the presence of DCMU on the inactivation rate of the reaction $H_2O \rightarrow$ SiMo due to a photoinhibitory treatment (time of PI). SiMo (0.1 mM) was added to the thylakoid membranes after the cessation of the photoinhibitory treatment. Oxygen evolution was assayed 2 min after the addition of SiMo. Bars represent standard deviation (n = 3-4)

Fig. 5.7. Effect of a photoinhibitory treatment on photosynthetic control at pH 6.4 and pH 7.6. Electron transport activity ($\mu mol(O_2).mg^{-1}(Chl).h^{-1}$) was measured from $DCPIPH_2$/ascorbate to MV in the absence (circles) and presence (diamonds) of 5 μM gramicidin. The experiment was performed at pH 6.4 (open symbols) and 7.6 (filled symbols).

protons through the membrane. For these experiments the reaction from $DCPIPH_2$/ascorbate to MV was chosen. Uncoupled e2lectron transport rate between this donor-acceptor couple decreased during a photoinhibitory treatment but this system was far less sensitive to photoinhibition than PSII-mediated electron transport (compare Fig. 5.1c and Fig. 5.7 (diamonds)). The $DCPIPH_2$/ascorbate reaction is inhibited by a low lumen pH and an increased proton permeability of the thylakoid membrane is reflected by an increase of the electron transport rate in the absence of an uncoupler. Fig. 5.7 shows that at pH 7.6 the electron transport rate in the absence of an uncoupler starts to increase as a consequence of the photoinhibitory treatment after a lag period of 10 min and after 25 min the rate has almost doubled. At pH 6.4 the lag period is shorter (about 5 min) and the increase of the electron transport rate in the absence of an uncoupler is larger. After a photoinhibitory treatment of 20 min the electron transport rate has almost tripled. The

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data indicate that after a photoinhibitory treatment of 15 min at pH 6.4 the uncoupled electron transport rate increases somewhat again. This might be explained if it is assumed that at this pH the addition of gramicidin did not dissipate the whole pH gradient and that the photoinhibitory treatment is a more effective uncoupler than gramicidin. It is further interesting to note that at both pH values electron transport in the presence of an uncoupler is not further inhibited after the end of the lag period.

Discussion

Use of SiMo
Several authors have used SiMo as an artificial electron acceptor to get information on the mechanism and site of photoinhibition (Kyle *et al.* 1984, Kyle *et al.* 1985, Cleland and Critchley 1985, Cornic *et al.* 1985, Richter *et al.* 1990). However, in most of these papers the experimental procedure has been described in insufficient detail to judge if all pitfalls were avoided. One characteristic that all of the cited experiments have in common is the use of DCMU to prevent electron flow to PSI. As demonstrated in the previous Chapter the use of DCMU complicates the use of SiMo considerably. To allow SiMo to displace DCMU from its binding site it has to be incubated with the inhibited thylakoids for at least a minute. However, the presence of reduced components, formed during the photoinhibitory treatment, will cause reduction of part of the added SiMo before the start of the measurement. This influences the measured kinetics (Schansker and Van Rensen 1993). A lower concentration of oxidized SiMo will shift the equilibrium between DCMU and SiMo-binding, lowering the measured electron transport rate (see Chapter 4). Because of the above mentioned reasons it is difficult to compare our results to those reported in the literature.

Comparison of $H_2O \rightarrow$ SiMo and $H_2O \rightarrow$ FeCy measurements
We have compared inactivation of SiMo-reduction with inactivation of FeCy-reduction to study the inactivation of PSII. This approach is only valid if in both cases PSII is rate limiting for electron transport. Measurements were done with thylakoids uncoupled either by NH_4Cl or gramicidin, systems in which PSII is expected to limit electron transport (Heber *et al.* 1988). Another difference is that the reaction rate for FeCy-reduction was two times higher than that for SiMo-reduction. However, as mentioned in the Results-section, under our circumstances SiMo binds only to about 50% of the PSII reaction centers (see also Chapter 4). This means that the differences in electron transport rates between both systems are relatively small, making the results more comparable. In neither system an appreciable lag in the inactivation was observed, also indicating that there is no rate limitation other than PSII. In conclusion, the comparison of the FeCy and the SiMo data seems to be valid.

Primary site of photoinhibition

The data in Fig. 5.1 indicate that both electron transport from H_2O to Q_A as well as electron transport from Q_A to the plastoquinone pool are sensitive to a photoinhibitory treatment. However, Fig. 5.1 also indicates that electron transport from Q_A to the plastoquinone pool is far more sensitive to light than electron transport from H_2O to Q_A. The conclusion that the primary site of photoinhibition is located between Q_A and Q_B is supported by *in vivo* data from Cyanobacteria (Kirilovsky *et al.* 1988 and 1990), *Chlamydomonas* (Kyle *et al.* 1984, Gong and Ohad 1991, Zer *et al.* 1994) and spinach (Briantais *et al.* 1992). In other papers the stabilization of charge separation is pinpointed as the primary site, meaning that Q_A does not function properly anymore (Vass *et al.* 1988, Van Mieghem *et al.* 1989, Farineau *et al.* 1990, Styring *et al.* 1990). This site was often explained by assuming double reduction of Q_A. As will be discussed later (see Chapter 8) several lines of evidence can be found in the literature that indicate that Q_A double reduction does not occur under aerobic conditions.

Acceptor and donor side inactivation

The data in Fig. 5.2 indicate that a dose response relationship exists for both the inactivation of FeCy- and SiMo-mediated electron transport. SiMo accepts electrons from Q_A and under saturating irradiances is relative insensitive to changes in the Q_B-region, while FeCy-mediated electron transport is sensitive to changes in the Q_B-region. This could mean that inactivation of FeCy-reduction is a measure for acceptor side inactivation and SiMo-reduction is a measure for donor side inactivation. In this case there are two possible interpretations: (1) there is a causal relationship between acceptor side inactivation and donor side inactivation in the sense that inactivation of the acceptor side makes the donor side more sensitive to light or (2) acceptor and donor side inactivation are two independent processes each with their own quantum yield of inactivation. The pH-dependence of both reactions is not the same (Fig. 5.3) and the data in Fig. 5.6 demonstrate that the presence of DCMU has no effect on the induction of donor side inactivation. These two observations favor the independence of both inactivation reactions. This independence is also supported by the observation of several authors that donors to PSII were able to overcome part of the photoinhibitory damage (maximally 10-20%) (Barenyi and Krause 1985, Bradbury and Baker 1986).

An alternative explanation for inactivation of SiMo-mediated electron transport would be a degradation of the D1-protein which would also prevent charge separation. However, there are three arguments that seem to exclude this option. Addition of DCMU which is known to prevent or at least delay the degradation of the D1 protein (Mattoo *et al.* 1984, Kyle *et al.* 1984, Kuhn and Böger 1990) does not prevent the inactivation of SiMo-mediated electron transport (Fig. 5.6). Secondly, the temperature dependence of D1 degradation favors an enzymatic degradation of the D1 protein (Aro *et al.* 1990). An enzymatic reaction is expected to saturate, certainly at the high irradiances that were used

in our experiments, but this was not observed (Fig. 5.2). In the third place, SiMo-mediated electron transport is more sensitive to light at both low and high pH values while for an enzymatic reaction the pH-dependence is expected to follow an optimum curve.

In conclusion, inactivation of SiMo-mediated electron transport is a measure for donor side inhibition and inactivation of FeCy-mediated electron transport is a measure for acceptor side inhibition. The data favor independence of donor and acceptor side inactivation.

Mechanism of photoinhibition
The working hypothesis of this thesis is that the loss of bicarbonate from its binding site is the primary event in photoinhibition. The role and presence of bicarbonate in PSII is a very elusive subject of study. At the moment there are no direct unambigual methods to study this phenomenon. On the basis of what is known about the characteristics of bicarbonate it is possible to make some predictions and test these experimentally. One of these predictions is that loss of bicarbonate should be facilitated below pH 7 (Stemler 1979, Stemler and Murphy 1983, Diner and Petrouleas 1990). In Fig. 5.4 this hypothesis was tested and confirmed. FeCy-mediated electron transport that depends on the presence of bicarbonate (sensitive to a formate treatment (Blubaugh and Govindjee 1988)) showed a marked increase of the rate of photoinhition as the pH of the medium was lowered below 7. SiMo-mediated electron transport that under saturating light is not sensitive to the presence of bicarbonate (not sensitive to a formate treatment (Blubaugh and Govindjee 1988)) did not show this type of pH-dependence but showed a broad optimum around pH 6.7 were SiMo-mediated electron transport was the least sensitive to photoinhibition. The irradiance dependence of photoinhibition (Fig. 5.2) indicates that for both SiMo- and FeCy-mediated electron transport there is a dose response relationship between irradiance level and inactivation. This cannot be associated with the photochemical turnover of PSII as the electron transport rate is very low in the absence of electron acceptors and the highest irradiances used would be super saturating.

Effect of photoinhibition on SiMo binding and reduction
Bradbury and Baker (1986) made a series of irradiance response curves of chloroplasts photoinhibited to varying degrees, measuring whole chain electron transport from $H_2O \rightarrow$ MV in the presence of an uncoupler. They observed that the irradiance necessary to halfsaturate this reaction (K_m) was not affected by the photoinhibitory treatment. Repeating the same experiment for the partial reaction $H_2O \rightarrow$ SiMo at pH 7.6 (Fig. 5.5) we observed a strong effect on the K_m (5.8 fold increase). In both cases PSII activity determines the rate (Heber *et al.* 1988). The difference can only be explained by assuming that the photoinhibitory treatment affects SiMo-binding and therefore that the photoinhibitory treatment affects the conformation of the acceptor side in some way.

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DCMU

There is a mix of results available on the protective effect of DCMU against photoinhibition. Satoh (1970) found that the presence of an inhibitor of PSII prevents inactivation of PSI but stimulated inactivation of PSII. Cornic and Miginiac-Maslow (1985) measuring SiMo activity and Mishra *et al.* (1991) monitoring the F_v/F_m found a smaller decline of these parameters if the photoinhibitory treatment was performed in the presence of DCMU. On the other hand Nedbal *et al.* (1986) measuring electron transport from H_2O to BQ after washing the thylakoids twice to remove DCMU found no protection against inactivation. Our results (Fig. 5.6) indicate that DCMU is neutral towards donor-side inhibition and give no information on damage to the acceptor-side.

Effect of a photoinhibitory treatment on the proton permeability of thylakoid membranes

As shown in Fig. 5.7 photosynthetic control is affected by photoinhibition. After a photoinhibitory treatment of 25 min at pH 7.6 or 12 min at pH 6.4 addition of an uncoupler does no longer stimulate the rate electron transport from $DCPIPH_2$/ascorbate → MV. Apparently, the lumen pH has lost its regulatory function. After 10 minutes of photoinhibitory treatment at pH 7.6 and 5 min at 6.4 (lag times of Fig. 5.7) about 70% of PSII activity was lost (Fig. 5.4). It seems that at both pH values a comparable extent of PSII inactivation is needed before proton permeability is affected (the electron transport rate in the absence of an uncoupler is stimulated). This observation is at variance with data of Tjus and Andersson (1993) who found no lag phase for stimulation and additionally found that the effect on proton permeability occurred at an even faster rate than inactivation of PSII. The extent of the stimulation found in their study (twofold) is comparable to our results. One explanation for the data could be that in thylakoid membranes in which the majority of PSII reaction centers is inactivated the formation of radicals increases. These radicals could cause lipid peroxidation which would lead to an increased permeability of the thylakoid membrane to ions including protons. Alternatively, the photoinhibitory treatment could affect the properties of ATPase and other ion channels leading to proton slip (Braun *et al.* 1991), though these authors observed this effect at basic pH values while we see an increase of the effect after lowering the pH of the medium. In conclusion, the photoinhibitory effect on the proton permeability of the membrane is suggested to be caused by radical induced lipid peroxidation. The radicals are likely to be produced by the inactivated PSII reaction centers. This conclusion seems to be in accordance with the measurements of Hideg *et al.* (1994a,b) who observed an increased production of singlet oxygen after the inhibition of PSII.

6 Effect of the substitution of glycine 215 by tryptophan in the D2 protein of *Synechocystis* sp. PCC 6803 on the characteristics of Photosystem II

Abstract

A Photosystem II (PSII) acceptor side mutant of *Synechocystis* sp. PCC 6803 was characterized to investigate the cause of its increased sensitivity to light. The substitution of glycine 215 by tryptophan in the D2 protein of PSII (G215W strain) affects PSII in several ways. G215W is more sensitive to formate than the reference strain. The formate concentration needed to inhibit the oxygen evolution rate by 50% was 1.6-times lower in G215W than in the reference strain. The formate-sensitive part of the fast component of the fluorescence decay curves of G215W was 10-times more sensitive to formate than in the reference line.

G215W is 4-times more sensitive to light than the reference strain. The only major effect of the mutation on the performance of PSII that was observed is the higher sensitivity of PSII to formate compared to the reference cells. A looser binding of bicarbonate to PSII as reflected by a higher sensitivity to formate is proposed as an explanation of the increased sensitivity of G215W to light.

Introduction

The cyanobacterium *Synechocystis* sp. PCC 6803 is used extensively for studies of the function of individual amino acids in PSII. A large number of site directed mutations in both the D1 and D2 proteins of PSII have been made (among others Diner and Nixon 1992, Kless *et al.* 1993, Mäenpää *et al.* 1993, Ohad and Hirschberg 1992, Philbrick *et al.* 1991, Vermaas *et al.* 1994). In Chapter 3 a relation between the loss of bicarbonate and the occurrence of photoinhibition has been postulated. One way to test this hypothesis would be to study the relation between properties of mutants with mutations in the

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vicinity of the non-heme iron (because of its proximity to the binding site of bicarbonate (Govindjee and Van Rensen 1993)) and the sensitivity of these mutants to light. One of these mutants is G215W. In this mutant one copy of the *psb*D-gene coding for the D2 protein has been deleted and a site-directed mutation was introduced in the other copy to mutate the glycine residue at position 215 of the D2 protein to a tryptophan. This glycine is a neighbor of one of the histidines that bind to the non-heme iron. Changing the histidines themselves severely affects the properties of PSII. Changing the histidine at position 214 of the D2-protein into an asparagine has been shown to prevent the assembly of PSII (Vermaas *et al.* 1987). Changing the histidine at position 268 of the D2-protein into a glutamine does not prevent assembly, but the assembled PSII-centers no longer produce oxygen (Vermaas *et al.* 1994). The effects of these two mutations are so drastic that such mutants cannot be used for physiological studies. Glycine 215 has also been exchanged for several amino acids other than tryptophan (Vermaas, unpublished data) but these changes were observed to yield photosynthetically inactive cells. G215W is more sensitive to light than the wild type in spite of a high initial electron transport rate (Van der Bolt and Vermaas 1992). EPR-measurements indicated that the $Q_A^-Fe^{2+}$-signal in the G215W-strain is reduced on a Chl basis but the shape of the signal is similar to that in the wild-type (Kirilovsky *et al.* 1992), indicating little change in the properties of the non-heme iron. On the other hand, it was difficult to induce thermoluminescence bands in G215W (Vermaas, unpublished results).

Here, we characterize the effects of the G215W mutation on the characteristics of PSII to investigate the higher sensitivity of this mutant to light. G215 is positioned close to the Q_A-binding site and the non-heme iron. As a consequence effects on charge stabilization and bicarbonate binding are possible. PSII of G215W was characterized using several fluorescence techniques and oxygen evolution measurements, and was compared with the PSII characteristics of a reference strain: *psb*DII⁻. The strain *psb*DII⁻, like G215W lacks the second copy of the *psb*D-gene. Fluorescence induction was measured to study the reduction kinetics of Q_A and fluorescence decay kinetics to determine the fate of the electron on Q_A^-. Formate was used to study effects of the mutation on the binding environment of bicarbonate and the non-heme iron.

Materials and methods

The mutants

The *psb*DII⁻-mutant and the G215W-mutant of *Synechocystis* sp. PCC 6803 were constructed as described by Vermaas *et al.* (1990a,b). Both strains were propagated at 30 °C and at an irradiance of 70 μmol.m⁻².s⁻¹ in liquid BG-11 medium (Rippka *et al.* 1979) to which 5 mM glucose was added. The strains were kept on plates under the same conditions as the liquid cultures. The plates contained BG-11, 0.3% w/v sodium thiosulfate, 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)/NaOH

(pH 8.0), 1.5% (w/v) Difco agar, 5 mM glucose, 25 μM atrazine, 20 μg.ml^{-1} spectinomycin and in the case of G215W 10 μg.ml^{-1} kanamycin. Cells were harvested when the OD_{730} was between 0.5 and 1.0. The Chl concentration was determined after methanol extraction according to the method of Tandeau de Marsac and Houmard (1988). The cells were collected by centrifugation (10 min at 7000 rpm) and subsequently resuspended at a concentration of 10 μg Chl.ml^{-1} in BG-11 medium set to pH 7.0 with 25 mM Hepes/NaOH. Cells were kept at room temperature.

In *psb*DII$^-$ one of the two copies of the gene coding for the D2 protein was deleted. The G215W-mutant also contains only one copy of the *psb*D-gene, but was also mutated at position 215. As we were only interested in the effects of the mutation in G215, *psb*DII$^-$ was used as a reference strain.

Oxygen evolution measurements
Initial oxygen evolution rates were measured using a Gilson oxygraph. Cells suspended in BG-11 medium buffered with 25 mM Hepes/NaOH (pH 7.0) were used for the experiments. During the measurements the cells were kept at a temperature of 25 °C. Light from a Xenon lamp (Oriel) was filtered through 17 cm water before reaching the reaction vessel. The measuring irradiance was 1500 μmol.m^{-2}.s^{-1} unless stated otherwise. Variation in irradiation was obtained using neutral density filters.

Fluorescence induction curves
Fluorescence induction curves were measured using a PAM-fluorometer (Walz, Effeltrich Germany). The light source for the induction experiments were the LEDs of the PAM 102-unit. Data acquisition was handled by the DA 100 data acquisition system (Walz, Effeltrich, Germany)

Fluorescence decay measurements
Cells at a concentration of 10 μg Chl.ml^{-1} were put in the measuring cell of a PAM-fluorometer and kept in the dark for several minutes before the measurement was started. A measurement consisted of a single turnover flash produced by a XST103 Xenon flash lamp (Walz). For every experiment 10 fluorescence decay curves were averaged. The time interval between flashes was 20 s. Data sampling and measurement averaging was done by the DA 100 data acquisition system at a sampling rate of 1 data point per 25 μs. To reduce the number of data points 4 measuring points were averaged. The amplitudes and halftimes of the decay components were determined, assuming exponential decay. The decay curve was plotted semilogaritmically. The slowest component was determined by linear regression and subtracted from the rest of the curve. This procedure was repeated till all components were determined.

Formate treatment
In the case of formate treatments, cells were incubated in the dark for 10 minutes with different concentrations of formate, before the measurements were carried out.

Herbicide binding experiments
The herbicide binding experiments were carried out with radioactively labeled DCMU as described by Vermaas *et al.* (1990c).

Results

Photosystem II content and activity
One of the effects of the mutation in the strain G215W might have been that the number of PSII reaction centers relative to the number of PS I reaction centers had changed. The number of PSII reaction centers on a chlorophyll basis in *psb*DII⁻ were compared with that in G215W by measuring diuron binding. Vermaas *et al.* (1990b) reported 550 Chl per DCMU-binding site in the wildtype and 1200 Chl per DCMU-binding site in G215W. We found 1440 Chl per DCMU-binding site for *psb*DII⁻ and 1370 in G215W. These data suggest that the G215W-mutation has little effect on the PSII-content of the cells on a Chl-basis. The binding constant of DCMU, K_D, for *psb*DII⁻ and G215W were measured to be 27 and 31 nM, respectively, while Vermaas *et al.* (1990b) found a value of 15 nM for the wildtype and 16 nM for G215W. In Fig. 6.1 the binding experiments are illustrated. The binding data indicate that the mutation in G215 has neither an effect on the number of PSII reaction centers on a chlorophyll basis nor on the affinity of the reaction centers for DCMU.

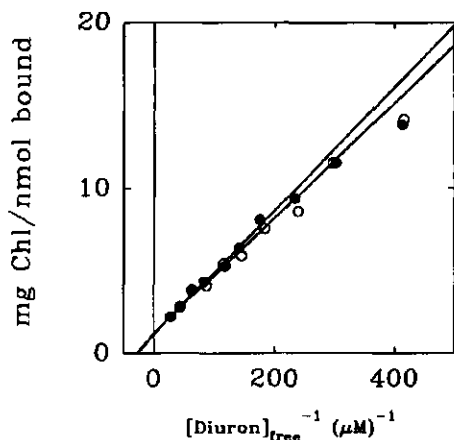*Fig. 6.1. Double reciprocal plots of [¹⁴C] diuron binding to cells of psbDII⁻ (filled circles) and G215W (open circles). The intersect with the Y-axis is proportional to the number of chlorophylls per PSII complex. The intersect with the X-axis is the inverse value of the diuron dissociation constant in vivo. Regression lines were calculated using the non-inverse data.*

The maximum electron transport rate of G215W measured was 265 μmol(O_2). mg^{-1}(Chl).h^{-1}. Using the number of PSII reaction centers of 1 per 1370 Chl molecules and assuming a M_r(Chl) of 820, it can be calculated that 1 mg Chl equals about 890 pmol PSII. One turnover of PSII is defined here as the time needed to reduce 1 plastoquinone molecule. According to this definition 1 molecule of O_2 is released for every 2 turnovers of PSII. From this it is calculated that every PSII has to turnover once per 6.1 ms to produce 265 μmol(O_2).mg^{-1}(Chl).h^{-1}. The same calculation for *psb*DII$^-$ (maximum electron transport rate of 460 μmol(O_2).mg^{-1}(Chl).h^{-1}) yields one turnover per 3.3 ms. This means that the turnover rate of whole chain electron transport is about 2 times slower in G215W compared to *psb*DII$^-$.

Fluorescence induction curves
The fluorescence induction curve in *psb*DII$^-$ is characterized by a fast rise to the I-level, followed by a second rise to the P-level that is completed within a few ms. Subsequently fluorescence declines again (Fig. 6.2A). In G215W a fast rise to the I-level is also observed, but the subsequent rise to the P-level is a very slow process, taking more than 50 s (Fig. 6.2B). Figure 6.2 also indicates that the F_o-level of G215W is 23% higher than
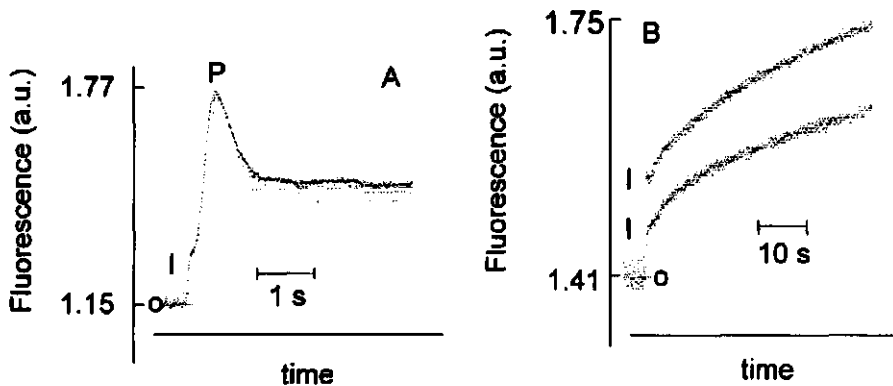
Fig. 6.2. Fluorescence induction curves of cells of psbDII$^-$ (A) and G215W (B). The actinic irradiance for psbDII$^-$ was 190 μmol.m^{-2}.s^{-1}; for G215W: 45 μmol.m^{-2}.s^{-1} (lowest trace) and 190 μmol.m^{-2}.s^{-1} (upper trace). Fluorescence was recorded during the first 5 s (psbDII$^-$) and 50 s (G215W) after turning on the actinic light. Only the variable fluorescence is plotted, it did not increase much after this 50 s in G215W. F_o and the maximal fluorescence level (P-level in psbDII$^-$) are indicated on the Y-axis. Fluorescence induction follows an O-I-D-P kinetics. In the case of G215W it takes more than 50 s to reach the P-level.

of *psb*DII⁻.

Fluorescence decay kinetics

In Fig. 6.3 fluorescence decay curves are shown of *psb*DII- and G215W-cells. In Table 6.1 the halftimes of the decay components and their amplitudes are presented. The different components of the decay curve are a manifestation of the different ways in which Q_A^- can be reoxidized. The fast component is a mix of electron transfer from Q_A^- to
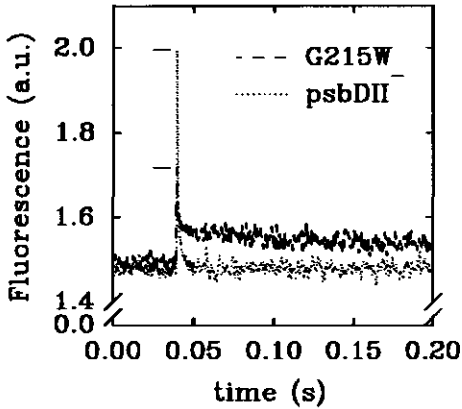
Fig. 6.3. Fluorescence decay curves of psbDII and G215W. Fluorescence changes after a saturating single turnover flash were recorded for 0.5 s of which 0.2 s is plotted in Fig. 6.3. Every trace is an average of ten experiments spaced 20 s apart.

Q_B and Q_B^-. The ms component is thought to signify electron transfer in a PSII with an empty Q_B-pocket. This reaction is limited by the binding of plastoquinone to the Q_B-site (Crofts and Wraight 1983). In stacked thylakoids protonation of $Q_B^=$ may become the limiting step for the exchange of plastoquinone (Jahns and Junge 1992). There exists an equilibrium between Q_A^- and Q_B^- (1:20 in higher plants) (Diner 1977 and Robinson and Crofts 1983). This means that about 5% of Q_A^- can recombine with the donor side. This recombination is the slow component in Table 6.1. Amplitude and halftime of the fast

Table 6.1. Amplitudes (% of total signal) of the different fluorescence decay components of psbDII and G215W after one single turnover flash. The decay halftimes of the different components are indicated in brackets.

	*psb*DII⁻	G215W
fast component	57 (300 µs)	57 (300 µs)
ms component	42 (1.5 ms)	17 (2 ms)
slow component	1.4 (0.5-1.5 s)	26 (200-400 ms)

component are comparable in both strains. The main difference seems to be that the amplitude of the slow component in G215W has increased at the expense of the ms component compared to psbDII⁻ (Table 6.1).

Effect of formate on oxygen evolution and fluorescence decay kinetics
In Fig. 6.4 the oxygen evolution rate is plotted as a function of the formate concentration. The data indicate that G215W is more sensitive to formate than psbDII⁻. From the data in Fig. 6.4 it is calculated that 1.6-fold less formate is needed in G215W to inhibit electron
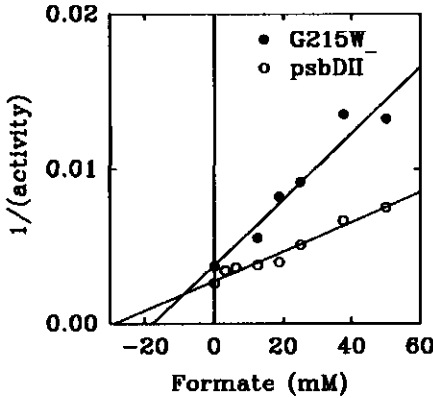
Fig. 6.4. Effect of formate on the oxygen evolution rate. The formate inhibition curves of psbDII⁻ and G215W are plotted as Dixon plots.

transport by 50% than in psbDII⁻.

In Figs. 6.5A and B the amplitudes of the different fluorescence decay components in both G215W and psbDII⁻ are plotted as a function of the formate concentration. In both strains the fast component declines from 60% in the absence of formate down to 20-25% in the presence of formate. However, in G215W this decline is already completed in the presence of 10 mM formate while in psbDII⁻ 60-70 mM is needed to reach the same level. In both strains part of the fast component (20-25% of the total amplitude) is insensitive to formate. In psbDII⁻ the ms component disappears (above 50 mM formate this component is no longer detectable). As the amplitude of the fast and ms-components decline, two formate induced components of 10-20 ms and 100-300 ms appear. The extent and the kinetics of the changes in the amplitudes do not allow a conclusion about shifts from one component to another. In G215W formate induces a 40-60 ms component. The increase of this component is complementary to the decrease of the fast component. Formate addition has little effect on the ms and slow component in G215W. Comparing the formate concentrations needed to inhibit oxygen evolution by 50% with the formate dependence of the fluorescence decay kinetics could indicate which decay component is associated with oxygen evolution. In psbDII⁻, the 29 mM formate needed to inhibit oxygen evolution by 50% (Fig. 6.4) corresponds nicely to the formate concentration (30-
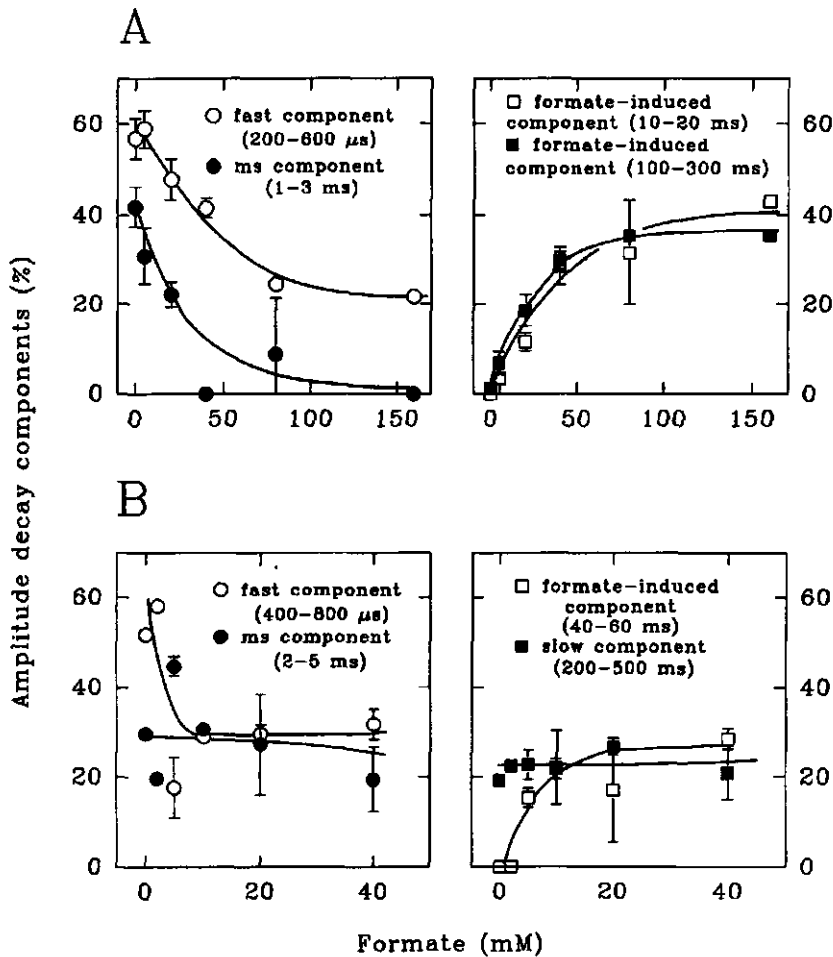
Fig. 6.5. *Effect of formate on the amplitudes of the different fluorescence decay components in psbDII (A) and G215W (B). Fluorescence decay curves as illustrated in Fig. 6.3 were measured in the presence of different formate concentrations. The cells were dark incubated with formate before the start of the measurement. The slow component in formate treated cells of psbDII was beyond the detection limit.*

oxygen evolution by 50% (Fig. 6.4) corresponds nicely to the formate concentration (30-40 mM) at which the amplitudes of the fast and ms components are reduced by 50% (Fig. 6.5A). In G215W, less formate is needed to inhibit the fast component by 50% (~5 mM) than for the same inactivation of the oxygen evolution rate (18 mM). It should be noted

than in *psb*DII⁻.

Formate may (El-Shintinawy *et al.* 1990) or may not (Srivastava *et al.* 1995) have an effect on the donor side of PSII. In order to test whether high formate concentrations affect charge separation under our conditions the F_v/F_m-values for all fluorescence decay measurements were determined and plotted against the formate concentration (data not shown). The treatment of *Synechocystis* cells with formate had little effect on the ratio between variable and maximum fluorescence induced by a single turnover flash. This indicates that the added formate concentrations had little effect on charge separation.

Sensitivity to light
In Fig. 6.6 the effect of the duration of a photoinhibitory treatment on *psb*DII⁻ and G215W is illustrated. From Fig. 6.6 and comparable experiments with other photoinhibitory photon flux densities rate constants for the inactivation of oxygen evolution of *psb*DII⁻ and G215W were determined. In Fig. 6.7 these rate constants are plotted as a function of the irradiance. In G215W the rate constants were determined at 4 different photon flux densities. As a comparison data for *psb*DII⁻ were also plotted, although in this case rate constants of inactivation at only two irradiances were available.
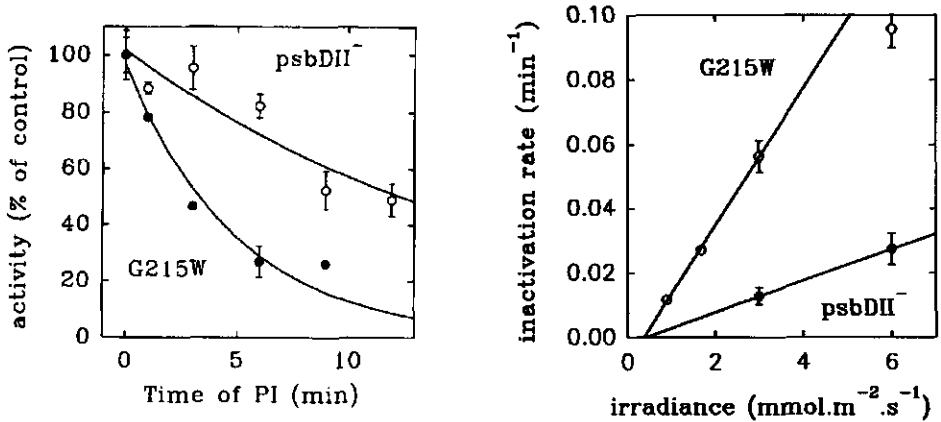
Fig. 6.6. Effect of the duration of a photoinhibitory treatment (PI; 6000 $\mu mol.m^{-2}.s^{-1}$) on the initial rate of oxygen evolution of cells of psbDII⁻ and G215W. Initial oxygen evolution was measured 2 min after cessation of the photoinhibitory treatment. The measuring irradiance for oxygen evolution was 1500 $\mu mol.m^{-2}.s^{-1}$.

Fig. 6.7. The rate constants for the inactivation of oxygen evolution of psbDII⁻ and G215W as a function of the photoinhibitory irradiance. Rate constants were derived from Fig. 6.6 and comparable experiments at other photon flux densities.

For G215W a linear relationship between irradiance and the rate of inhibition was observed. It was assumed that for *psb*DII⁻ also a linear relationship exists. A linear relationship between irradiance and the rate of inactivation indicates that a dose-response relationship exists between the photoinhibitory irradiance and the halftime of inactivation for oxygen evolution. The intersect of the extrapolated line with the x-axis yields information on the irradiance at which inactivation and repair balance each other. However, there are too few data to determine this irradiance with any precision.

Effect of the measuring irradiance on the rate of inactivation of oxygen evolution
Van der Bolt and Vermaas (1992) described a fast method for the determination of the sensitivity of *Synechocystis* cells to light. In this method cells are photoinhibited and at the same time oxygen evolution is measured. The time point at which the oxygen evolution rate has dropped by 50% is taken in this case as the halftime of inactivation[1].
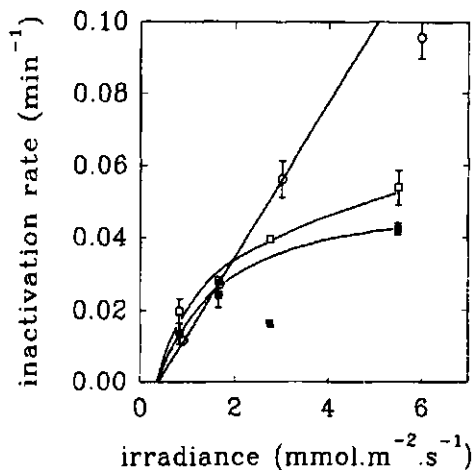
Fig. 6.8. Effect of the measurement irradiance on the relationship between the rate constant of inactivation of oxygen evolution and the photoinhibitory irradiance in G215W. Measurements were carried out as described in the text. The measuring points determined according to the method of Vermaas and Van der Bolt (1992) are plotted as squares; measurements done in the absence of the DMBQ/FeCy couple (open squares) and measurements done in the presence of the DMBQ/FeCy couple (filled squares). To demonstrate the difference with the other method the G215W data from Fig. 6.7 were plotted in the same figure (open circles).

The experimental setup implies that the photoinhibitory irradiance and the irradiance at which the oxygen evolution rate is measured are the same. To compare this method with the one described earlier in this Chapter the halftime of inactivation of oxygen evolution at 4 irradiance levels was determined in the presence or absence of DMBQ (Fig. 6.8) and

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[1] In experiments with *psb*DII⁻ the oxygen evolution rate stayed at a constant high value for times longer than the measuring times with G215W, indicating that substrate limitations played no role during the experiment.

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at 4 irradiance levels was determined in the presence or absence of DMBQ (Fig. 6.8) and the results were compared with Fig. 6.7. Both methods are only comparable if in *Synechocystis* cells the observed extent of photoinhibition is independent of the measuring irradiance. As demonstrated in Fig. 6.8 the two methods lead to deviating results. Using the second method a non-linear relationship between the photoinhibitory irradiance and the rate of inactivation is observed. That the method described by Van der Bolt and Vermaas (1992) by itself is valid is demonstrated by the fact that when the measuring irradiances are comparable (~ 1500 μmol.m^{-2}.s^{-1}) both halftimes of inactivation are comparable. The discrepancy between both methods can be explained by assuming that the relationship between PSII inactivation and inactivation of the oxygen evolution rate depends on the measuring irradiance level. This irradiance dependence is caused by the fact that PSII limits electron transport only at non saturating irradiances (see Chapter 1).

Van der Bolt and Vermaas (1992) tried to avoid problems with the irradiance dependence of inactivation by the addition of the DMBQ/FeCy-couple. This combination of electron acceptors is thought to be able to measure PSII selectively (Vermaas *et al.* 1990b, Van der Bolt and Vermaas 1992). If this assumption is correct there should be a linear relationship between the extent of inhibition of PSII and the extent of inactivation of the oxygen evolution rate in the presence of DMBQ and FeCy. In that case a linear relationship between the photoinhibitory irradiance and the inactivation rate of oxygen evolution is expected. The slope of this line should be at least as steep as the slope of the line determined with the other method (Fig. 6.7). As observed in Fig. 6.8 neither of these criteria is met. There is no linear relationship between the photoinhibitory irradiance and the rate of inactivation of the oxygen evolution activity and if a line would have been drawn through these points the slope would have been less steep than the slope of the line in Fig. 6.7. The measurements done in the presence of DMBQ/FeCy are very comparable to those in the absence of DMBQ/FeCy indicating that at least in G215W this couple cannot be used to measure PSII selectively.

Effect of the DMBQ/FeCy couple on electron transport rates
To study the effects of DMBQ on electron transport in more detail irradiance response curves of the oxygen evolution rate in the presence and absence of the DMBQ/FeCy couple were made (Table 6.2). Although O_2 evolution at light saturation was unaltered, the irradiance needed to half-saturate oxygen evolution in *psb*DII$^-$ shifted from 170 μmol.m^{-2}.s^{-1} to 340 μmol.m^{-2}.s^{-1} in the absence and presence of DMBQ and FeCy, respectively. In G215W the differences were even larger and, in addition, in this case the presence of DMBQ and FeCy did affect the maximum oxygen evolution rate (Table 6.2). At saturating irradiances PSII is not rate limiting for whole chain electron transport. The observation that more light is necessary to half saturate electron transport in the presence of DMBQ might indicate that DMBQ occupies the Q_B-binding site but accepts electrons very slowly from PSII and acts as a kind of competitive inhibitor of PSII. As a

Table 6.2 Characteristics of the irradiance dependence of the oxygen evolution activity of psbDII⁻ and G215W cells in the presence and absence of DMBQ and FeCy.

	psbDII⁻		G215W	
	no additions	DMBQ + FeCy	no additions	DMBQ + FeCy
K_m (μmol.m^{-2}.s^{-1})	170	300	160	850
V_{max} (μmol(O$_2$).mg^{-1}(Chl).h^{-1})	460	455	200	135

of DMBQ and a higher turnover rate of these centers would be necessary before the rate limitation shifts from PSII to another part of the electron transport chain. The data suggest that DMBQ is an even worse electron acceptor (or a better inhibitor) of PSII in G215W than in *psb*DII⁻. The interpretation that DMBQ is a bad electron acceptor is supported by the existence of a D2-mutant of *Synechocystis* sp. PCC 6803 that has a normal electron transport activity in the absence of DMBQ but is fully inhibited in the presence of DMBQ (Ermakova and Vermaas, unpublished data).

The irradiance response data support the photoinhibition data on DMBQ. Both types of data suggest that DMBQ in G215W is not an effective shuttle between PSII and FeCy, because it is a bad PSII electron acceptor.

Discussion

Fluorescence induction curves
In cyanobacteria as well as in higher plants fluorescence induction follows an O-I-D-P-sequence. In *psb*DII⁻ the P-level is reached within a few ms whereas in G215W no indications for an I-P rise were found. After reaching the I-level a slow rise is observed in G215W but this rise is probably more related to state transitions as it was not sensitive to atrazine (not shown). In *psb*DII⁻ the fluorescence level declines again after reaching the P-level. In the wild type this decline is followed by a secondary rise (S-M rise) to a level much higher than the P-level (Mohanty and Govindjee 1973, Vermaas *et al.* 1994).

Fluorescence decay kinetics
The fluorescence decay kinetics of G215W and *psb*DII⁻ were determined to study the acceptor side of PSII. In higher plants there is no linear correlation between fluorescence and the concentration Q_A^-. However, in *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 6301 little energy transfer between open and closed reaction centers was observed and a good correlation between F_v and PSII-content (Philbrick *et al.* 1991,

Nixon and Diner 1992, Mullineaux and Holzwarth 1993). The absence of connectivity between PSII reaction centers means that non-linearity problems as mentioned by Robinson and Crofts (1983) are not encountered.

In G215W a halftime for the slow phase of 200-400 ms was found. This value compares well to the decay halftime in the presence of DCMU of 200-600 ms (Nixon *et al.* 1991, Phillbrick *et al.* 1991, Chu *et al.* 1994, Vermaas *et al.* 1994). In *psb*DII⁻ halftime values for the slow component were somewhat higher but the amplitude of the slow phase was so small that an accurate determination of the decay halftime was difficult.

With thermoluminescence it is possible to study recombination reactions between the acceptor and donor side of PSII. Recombination reactions are prevented by lowering the temperature below the activation energy. By increasing the temperature again above the activation energy the recombination reactions can take place. The B-band is observed when Q_B^- recombines with S_2. A shift of this band to lower temperatures means that the activation energy for recombination is lower and that in this case the equilibrium between Q_A^- and Q_B^- has shifted towards Q_A. Etienne *et al.* (1990) observed a negative correlation between the temperature at which the B-band occurs and the amplitude of the slow component (depends on the equilibrium between Q_A^- and Q_B^-, see above and Chapter 2). This negative correlation confirms that an increase of the amplitude of the slow component is due to a shift of the equilibrium between Q_A^- and Q_B^- towards Q_A^-. However, G215W might be a special case in this respect. It was observed that in G215W the amplitude of the slow component depends on the age of the cells (Vermaas, unpublished results). In young cells ($OD_{730} \sim 0.2$) the amplitude of the slow component was more than 60% whereas in older cells ($OD_{730} \sim 0.8$) this amplitude had declined to 5%. The data in Table 6.1 describe an intermediate case. The G215W cells were grown on glucose. As illustrated in Figs 6.4 and 6.5, G215W is more sensitive to formate and this might lead to the conclusion that a metabolite or metabolites from glucose are able to bind to PSII and affect PSII in the same way as formate does. As the culture ages the glucose is used up and this is reflected by a decrease of the amplitude of the slow component.

In metabolite-affected PSII reaction centers release of double reduced plastoquinone is a slow process which might explain the decrease of the amplitude of the ms component.

Effects of formate on fluorescence decay kinetics and oxygen evolution

The formate treatment caused a decrease of the contribution of the fast component. At the same time formate induced a component with a halftime of 40-60 ms in G215W and 100-300 ms in *psb*DII⁻ (Fig. 6.5). If it is assumed that the slow components reflect charge recombination in the presence of formate, the halftimes of these slow components are considerably shorter than in the absence of formate (compare with Table 6.1). The faster halftime in the presence of formate could indicate that the formate treatment causes a

destabilization of Q_A^- accelerating the recombination rate between Q_A^- and S_2.

Vernotte *et al.* (1995) studied the effect of formate on herbicide resistant mutants of *Synechocystis* sp. PCC 6714 with one or two mutations in their D1-protein. In these mutants the amplitude of the ms-phase was not affected by a treatment with 20 mM formate while the halftimes increased by a factor 1.5. In G215W formate had a comparable effect on the ms component. In *psb*DII⁻ no effect on the amplitude was observed whereas the decay halftime became about 5 times longer (Fig. 6.5).

Even at the highest formate concentrations there is a residual 20-25% of the fast component that seems to be insensitive to formate. The fast component is a mix of electron transfer from Q_A^- to Q_B and Q_B^-. The transfer to Q_B is insensitive to formate (Cao *et al.* 1991). The difference in formate sensitivity between these two electron transfer processes can explain the formate dependence of the decrease of the amplitude of the fast component. This would mean that before the start of the experiment ~35% of the PSII reaction centers were in the state $Q_AQ_B^-$ and ~25% percent in the state Q_AQ_B (Fig. 6.5).

In *psb*DII⁻ a correlation exists between the concentration of formate needed to decrease the amplitude of the fast and ms components by 50% and the concentration of formate needed for the inactivation of oxygen-evolution activity by 50% (compare Fig. 6.4 and 6.5A). In G215W this correlation is lacking. The fast component of the fluorescence decay curve of G215W is 10 times more sensitive to formate than the fast component in *psb*DII⁻. However, this does not translate into a 10 times lower concentration of formate needed in G215W to inhibit electron transport by 50%. On the contrary, this difference is in the case of the inactivation of the oxygen evolution activity by formate only a factor 1.6.

Sensitivity to photoinhibition

As observed earlier, cells of G215W are considerably more sensitive to photoinhibition than wild-type *Synechocystis* cells (Vermaas *et al.* 1990b, Van der Bolt and Vermaas 1992). In both *psb*DII⁻ and G215W the inactivation of PSII was a first order process (Fig. 6.6) and linear relations were found between the irradiance level and the rate of inactivation (Fig. 6.7). This means that in both strains a dose-response relation exists between irradiance and the rate of inactivation. At the two irradiances for which the rate of inactivation was determined in both strains it was found that G215W was inactivated 4 times faster than *psb*DII⁻ (Fig. 6.7).

One of the goals of this research was to find a correlation between functional changes as a consequence of the mutation and the 4-fold higher sensitivity to light. The fast component of the fluorescence decay curve in the mutant is about 10 times more sensitive to formate than in *psb*DII⁻. This might mean that bicarbonate is bound more loosely to mutated reaction centers which would favor the working hypothesis. Other experiments have to be designed to determine the reason for the higher sensitivity of G215W to light unambiguously.

7 Photosynthetic performance of photoinhibited pea leaves: a photoacoustic and fluorescence study

Abstract

The effect of photoinhibition on the performance of photosynthesis in pea leaves was determined by studying the relationship between the severity of a photoinhibitory treatment (measured as F_v/F_m) and several photoacoustic and chl a fluorescence parameters. The parameter F_v/F_m was a good indicator for the severity of photoinhibition in pea as there existed a linear relationship between decline of F_v/F_m and the potential oxygen evolution rate, determined by a photoacoustic method. This analysis demonstrated the presence of PSII reaction centers inactive in oxygen evolution and less sensitive to photoinhibition. The parameter q_P (ratio between open and total reaction centers) was stable over a broad range of F_v/F_m-values. Correcting q_P for the population of inactive centers unveiled an increase of q_P in severely inhibited pea leaves, indicating that the rate limitation of whole chain electron transport shifts from cyt b_6/f towards PSII as the severity of the photoinhibitory treatment increases. The data also showed that down to an F_v/F_m-value of 0.70 the excitation pressure on still active PSII reaction centers increased. Analysis of q_E (high energy quenching) demonstrated an initial increase of q_E probably associated with dephosphorylation of LHCII. Correcting q_E for PSII reaction centers inactivated by the photoinhibitory treatment indicated that there is only a small decrease of q_E as the severity of the photoinhibitory treatment increases. Analysis of q_I showed that the halftime of recovery of q_I increases steeply below an F_v/F_m of 0.70. This increase of the relaxation halftime correlates well with the decrease of the electron transport rate J and indicates that the supply of ATP starts to decrease. It also indicates that cyclic electron flow is not able to compensate for the decreasing supply of ATP.

Introduction

When plants are exposed to irradiances that are much higher than those they are adapted to, they will make use of several mechanisms to avoid or dissipate the excess energy. If these mechanisms fail the system will be damaged. Using fluorescence data it is often

difficult to distinguish between the effects of regulatory mechanisms and of damage. One way to separate these two aspects might be to look at the pattern of changes of different fluorescence and photoacoustic parameters as the severity of the photoinhibitory treatment increases. Regulatory mechanisms are expected to saturate, while damage is expected to proceed progressively as the severity of the treatment increases.

PSII and antenna-associated processes have been characterized by analyzing changes in q_P, q_N (q_E, q_T and q_I) and ϕ_P. Lokstein *et al.* (1993) used a combination of antenna size mutants and changes in the irradiance; Falk *et al.* (1994a,b) and Briantais (1994) used intermittent light grown plants as well as antenna size mutants; Ögren (1991) and Van Wijk and Van Hasselt (1993) used leaf discs inhibited with different irradiances.

In this chapter we describe the effects of photoinhibition on the photosynthetic performance of pea leaves at an actinic irradiance of 320 μmol.m^{-2}.s^{-1} by studying and analyzing several fluorescence and photoacoustic parameters after photoinhibitory treatments of variable length and intensity.

Materials and methods

Plant material
Leaves of 10 - 15 days old pea plants (*Pisum sativum* cv. Finale) grown under 200 μmol.m^{-2}.s^{-1} irradiance were used for the experiments.

Fluorescence measurements
Fluorescence measurements were done according to the protocol in Fig. 7.1 with a PAM-fluorescence meter. The actinic irradiance used was 320 μmol.m^{-2}.s^{-1}. Before the photoinhibitory treatment the leaves were given a saturating light pulse to determine F_o and F_m levels used for the calculation of q_N. After the photoinhibitory treatment the leaves were dark adapted for 15 min before starting the measurements. During this period probably some recovery occurs but this is ignored. After the period of dark adaptation F_o and F_m levels were determined again and F_v/F_m was calculated to determine the extent of photoinhibition. After the saturating pulse the actinic light was turned on and during a period of 700 s the fluorescence was allowed to reach a quasi steady state. The advantage of this protocol is that the fluorescence parameters were determined for relatively normal light conditions. A second advantage is, that the irradiance during the photoinhibitory treatment may vary without affecting the results. A limitation of the method is the need for a saturating pulse. Below an F_v/F_m-value 0.55-0.60 the number of active reaction centers has become so low that it is unlikely, that the light pulse is still completely reducing the PQ pool (no matter how high the irradiance). This means that an accurate
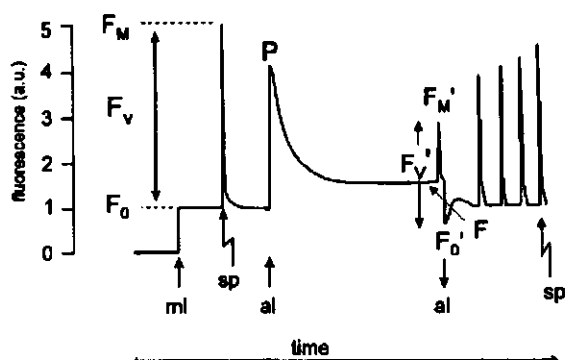
Fig. 7.1. Measurement protocol of the fluorescence experiments. 15 min after the end of a photoinhibitory treatment the measurement was started by determining F_o and F_m values. Subsequently, the actinic light (320 $\mu mol.m^{-2}.s^{-1}$) was turned on for a period of 700 s to allow the leaves to reach a quasi steady state (there will be a continuous recovery in photoinhibited leaves in the light). At the end of the 700 s F and F_m' are determined and after the actinic light is turned off, F_o'. Recovery of fluorescence was followed in the dark by giving saturating pulses after 100 s and every 200 s after the first pulse. The relaxation kinetics of fluorescence in the dark were used to determine q_E, q_T and q_I. Abbreviations: ml, measuring light; al, actinic light; sp, saturating pulse.

determination of the fluorescence parameters of the active centers is no longer possible. However, it is expected that measurements of the fluorescence emitted by inactive centers is not affected by this limitation, because of their low turnover.

Fluorescence parameters were calculated according to Van Kooten and Snel (1990). One of these parameters is q_P, which is a measure for the fraction of open reaction centers at a given actinic irradiance. When analyzing q_P, several assumptions were made. The amplitudes of F and F_m' are modulated by the ΔpH across the thylakoid membrane (q_E). A low lumen pH quenches these amplitudes. It was assumed that the differences in q_E at steady state conditions between the different treatments were small and had a relatively small effect on the amplitudes of F and F_m'. This would make the amplitudes of these parameters comparable at different F_v/F_m-values. With this assumption the parameter $F-F_o'$ (fluorescence emitted by closed reaction centers) was used as a measure for the number of closed reaction centers and the parameter $F_m'-F$ (fluorescence emitted by open reaction centers) as a measure for the number of open reaction centers.

After turning off the actinic light, several processes are reversed (among others the ΔpH across the thylakoid membrane is dissipated). Increases of F_v as a consequence of

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the reversion of these processes can be followed by calculating q_N as a function of the time after turning off the actinic light. In the experiments where fluorescence relaxation in the dark was followed for a longer time, q_N was analyzed according to Walters and Horton (1991). Using this analysis it is possible to distinguish at least three components making use of the differences in relaxation times of these components. The parameter q_E, associated with the presence of a low lumen pH, is characterized by a relaxation halftime of 10-20 s. The parameter q_T, associated with state transitions and possibly also containing another less well defined component, is characterized by a relaxation halftime of several min. The third component, q_I, is associated with resynthesis of the D1 protein and the recovery of photoinhibition and has a halftime of relaxation of several hours. For the calculation of q_E the relaxation of variable fluorescence during the first 100 s after turning off the actinic light was used. The parameter q_N is defined as:

$$q_N \approx 1 - \frac{(F'_M - F'_o)}{(F_M - F_o)}$$

The definition of q_N indicates that it is calculated relative to a reference fluorescence level. This means that the fast component of q_N, q_E, can be defined in two ways; either on the basis of F_m and F_o before the photoinhibitory treatment as done in Fig. 7.7a, or corrected for inactivated reaction centers (elimination of q_I) by using F_m and F_o determined 15 min after the photoinhibitory treatment as done in Fig. 7.7b (see also Van Wijk and Van Hasselt 1993).

Photoacoustic measurements
After dark adaptation leaf discs were cut out of the irradiated leaf and homogeneously punctured with a sharp needle to assist a good diffusion of oxygen out of the leaf independent of stomatal opening.

The experimental setup was as described by Snel *et al.* (1990) and Snel *et al.* (1992). System operation and data acquisition were done using a dedicated program PHOTACOS (author W.F. Buurmeijer). Measurements were done using red modulated light (142 Hz) of 280 μmol.m^{-2}.s^{-1}. The composite oxygen and heat signal was separated using a lockin amplifier as described by Snel *et al.* (1990). An example of a measurement is given in Fig. 7.2a. A saturating 700 ms pulse was given 1.3 s after turning on the actinic light (at t = 1, see Fig. 7.2a). During this pulse the modulated oxygen evolution rate declines to zero and the quadrature signal contains only a contribution of the heat-signal. The amplitude of the quadrature signal during the saturating pulse was used for the determination of the heat signal. In Fig. 7.2b the photobaric data (oxygen evolution) of Fig. 7.2a are plotted as a function of the fluorescence data of Fig. 7.2a. In Fig. 7.2b fluorescence and photobaric data of three different treatments were used. The last 1.3 s of
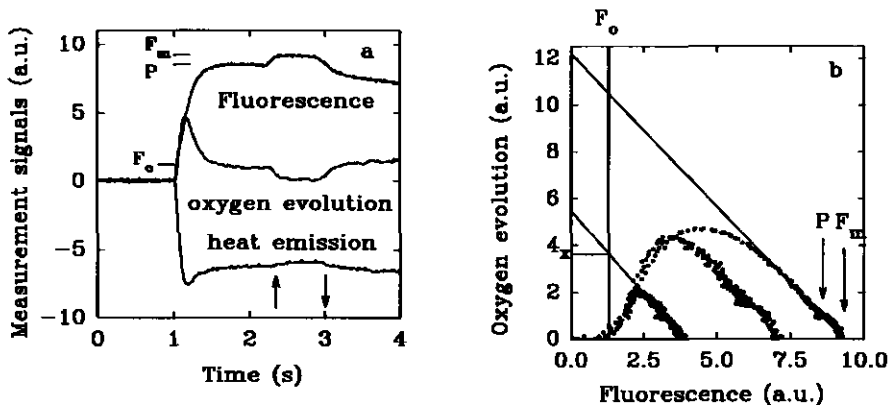
Fig. 7.2. Example of a photoacoustic experiment in which the fluorescence (PAM) and photoacoustic signals were detected simultaneously. a. measuring traces; the photoacoustic signal was resolved in an oxygen and a heat signal. A saturating 700 ms pulse was given 1.3 s after turning on the actinic light (indicated by arrows). b. oxygen evolution trace plotted as a function of the fluorescence trace for three different levels of photoinhibition. Lines were fitted to the points before the fluorescence levels off (cluster of points). After the saturating pulse oxygen evolution declines to 0 and the fluorescence reaches the F_m-level. Potential oxygen evolution was determined by extrapolation of the regression line to the F_o-level; x is the calculated potential oxygen evolution for the plot with F_v/F_m is 0.68.

the measurement were omitted to simplify the figure. The photobaric signal rises to a maximum value and subsequently declines again. However, the maximal photobaric signal is lower than the theoretical maximum because of diffusion limitations and the response time of the equipment, which was set at 30 ms. After reaching the maximum photobaric signal, a linear correlation exists between the decline of the photobaric signal and the rise of fluorescence as a consequence of the closing of PSII reaction centers. By extrapolating the linear part of the curve back to F_o (all reaction centers are oxidized) it is possible to calculate the potential oxygen evolution rate (Vredenberg *et al.* 1992).

Photoinhibitory treatment
Plants were taken out of the soil and put into an Erlenmeyer with water. The next step was to put the plants in front of a slide projector lamp. The light was filtered through a water filter before it reached the leaf. Additional protection of the plant against heating was provided by the cooling fan of the slide projector. Plants were illuminated from 5 up

to 30 min with irradiances from about 500 up to 5000 μmol.m^{-2}.s^{-1}. The severity of the treatment was determined after 15 min of dark adaptation by determining F_v/F_m. Wilted leaves were discarded.

In this paper the expressions inactive centers and inactivated centers are used. Inactive centers are defined here as PSII reaction centers that were already present in the leaf before the photoinhibitory treatment, emit variable fluorescence, but do not evolve oxygen at a modulation frequency of 142 Hz and are less sensitive to a photoinhibitory treatment than normal active PSII reaction centers. Inactivated centers are defined as PSII reaction centers loosing their photosynthetic competence as a consequence of the photoinhibitory treatment. It is assumed that they no longer emit variable fluorescence.

Results

Oxygen evolution

In Fig. 7.3 the results of the determinations of the potential oxygen evolution rate after a photoinhibitory treatment are plotted as a function of F_v/F_m belonging to that state. A linear relationship between both parameters is found. However, the line extrapolates to an F_v/F_m-value of about 0.44 and not to 0. The F_o-level was found to be almost constant as F_v/F_m-value declines. With the experimental F_o it is possible to calculate F_v at an F_v/F_m-value of 0.44. This F_v-value is equal to about 12% of F_v in control leaves and this percentage cannot be assigned to PSII reaction centers producing oxygen (Fig. 7.3). The relationship between F_v/F_m and the potential oxygen evolution can be used as a tool to determine the extent of the photoinhibitory damage. In the following figures the parameter F_v/F_m was used to titrate the effect of a photoinhibitory treatment on the measured fluorescence and photoacoustic parameters.
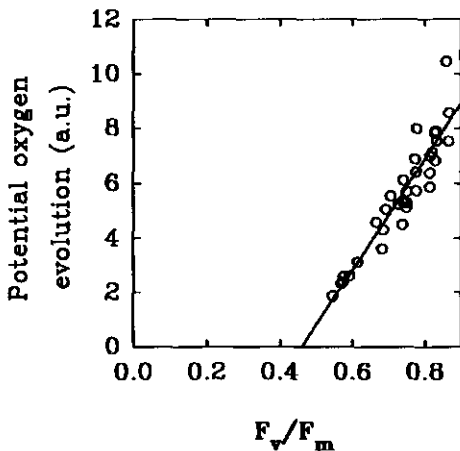
Fig. 7.3. Effect of a photoinhibitory treatment on the relationship between efficiency of PSII in the dark (F_v/F_m) and the potential oxygen evolution. The potential oxygen evolution was determined as described in Fig. 7.2b. The F_o and F_m-levels necessary for the calculation of F_v/F_m were determined separately before the the start of experiments that were illustrated in Fig. 7.2a.

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Photochemical quenching

The photochemical quenching (q_P) is the ratio between the fluorescence emitted by open PSII reaction centers (during a saturating pulse) and the fluorescence emitted by all PSII reaction centers at a certain actinic irradiance ($q_P = (F_m' - F)/(F_m' - F_o')$). In Fig. 7.4a, q_P after a photoinhibitory treatment is plotted as a function of F_v/F_m belonging to that state. In Fig. 7.3 it was observed that at an F_v/F_m-value of 0.44 all active reaction centers were inactivated. Inactive centers have a low turnover rate and hence it can be assumed that
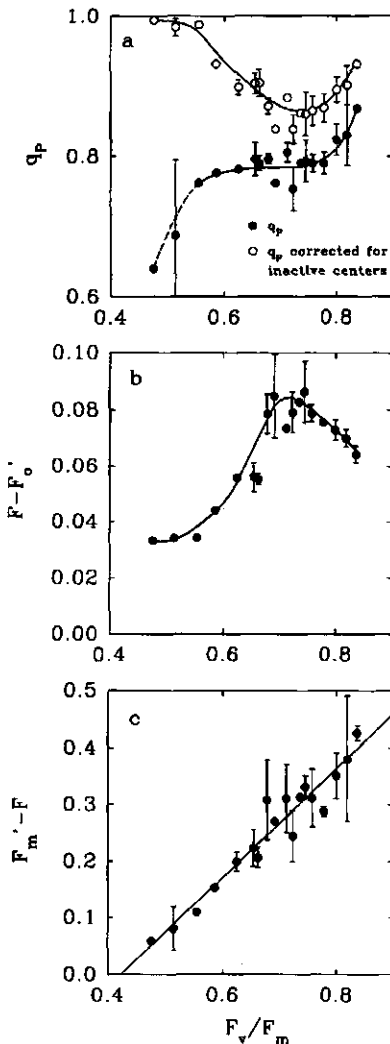
Fig. 7.4. Effect of a photoinhibitory treatment on the reduction level of the population of PSII reaction centers: a. Relationship between the severity of the photoinhibitory treatment expressed as F_v/F_m and the photochemical quenching q_P. Without (closed circles) and with (open circles) a correction for inactive centers (the method of correction is described in the text) b. Number of closed reaction centers (F-F_o') as a function of F_v/F_m. c. Number of open reaction centers (F_m'-F) as a function of F_v/F_m. The data in b and c were normalized to the F_v of a dark adapted control leaf. Bars represent the standard deviation (n = 2-6).

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they are in the closed state at 320 μmol.m^{-2}.s^{-1}. This means that q_P is expected to drop to 0 at an F_v/F_m-value of 0.44. As found by Öquist et al. (1992a), q_P is hardly affected by the photoinhibitory treatment over a broad range of F_v/F_m-values. One of the factors that affects q_P is the presence of inactive centers. As the number of active reaction centers decreases the relative contribution of the inactive centers to the fluorescence signal will increase and they will increasingly affect the calculation of q_P. To assess the effect of a photoinhibitory treatment on q_P of the active reaction centers it is neccesary to correct the data for the presence of inactive centers. One way to do this is to analyze the parameter $F-F_o'$ (fluorescence emitted by closed reaction centers) as a function of F_v/F_m. As the number of active centers declines a decreasing number of PSII reaction centers has to feed electrons to an unaltered number of PSI reaction centers. Consequently, the plastoquinone pool will become oxidized and the rate of Q_A reoxidation increases. Thus, the population of closed active centers is expected to decline to a value close to 0 as the number of active centers declines. The residual fluorescence can then be assigned to inactive reaction centers. In Fig. 7.4b $F-F_o'$ after a photoinhibitory treatment is plotted as a function of F_v/F_m. Looking at the pattern of changes it is observed that the number of closed reaction centers increases down to an F_v/F_m of 0.72 and declines below an F_v/F_m-value of 0.72. The decrease of the number of closed reaction centers is probably caused by a shift of the rate limitation of electron transport from the cyt b_6/f-complex and the Calvin cycle to PSII. This shift allows a higher reoxidation rate of the still active reaction centers and increases the number of open reaction centers at the expense of the number of closed reaction centers. Below an F_v/F_m of 0.60 $F-F_o'$ reaches a constant value of about 3.3% of F_v of a control leaf. This fluorescence is assigned to the inactive centers. By substracting this fluorescence from the measured F_m-F_o'-value (fluorescence emitted by all reaction centers) only the active centers are taken into account. The q_P-values corrected for the inactive centers as described here are plotted as a function of F_v/F_m as well (Fig. 7.4a). After an initial decline q_P increases again as F_v/F_m falls below 0.70.

In Fig. 7.4c $F_m'-F$ (a measure for the number of open centers) is plotted as a function of F_v/F_m. Open reaction centers are almost by definition active reaction centers. $F_m'-F$ declines as F_v/F_m declines and extrapolates to an F_v/F_m-value of 0.42. This value is very close to the one found for the photoacoustic experiment in Fig. 7.3. The parameter $F_m'-F$ like the parameter $F-F_o'$ is influenced by q_E-quenching. However, the fact that $F_m'-F$ declines almost linearly to 0 as the severity of the photoinhibitory treatment increases indicates that the effect of q_E-quenching is independent of the severity of the photoinhibitory treatment. This also gives an extra justification for the use of the parameter $F-F_o'$ as a measure for the number of closed reaction centers.

Electron transport rate
In Fig. 7.5 J (a measure for the electron transport rate) after a photoinhibitory treatment is plotted as a function of F_v/F_m belonging to that state. The definition of J is pfd*ϕ_P and
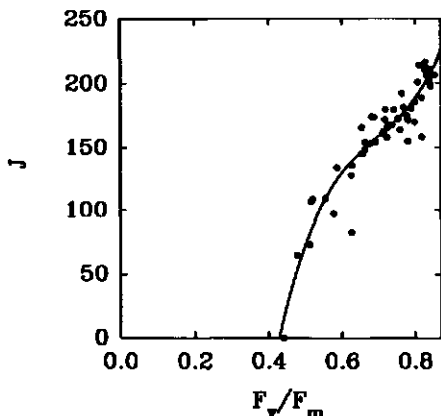
Fig. 7.5. Effect of a photoinhibitory treatment on whole chain electron transport. A measure for the electron transport rate (J) at 320 $\mu mol.m^{-2}.s^{-1}$ is plotted as a function of the severity of the photoinhibitory treatment expressed as F_v/F_m. At an F_v/F_m of 0.44 oxygen evolution was 0 (Fig. 7.3) and hence the electron transport rate (J) is also expected to be 0.

as in our experiments the photon flux density is kept constant, J and ϕ_P follow the same pattern. As F_v/F_m declines, J declines too. Again, J is expected to decline to 0 as F_v/F_m declines to 0.44. A large part of the decline of J can be attributed to centers inactivated by the photoinhibitory treatment, no longer emitting F_v-fluorescence but still emitting F_o-fluorescence. A contribution of less efficient, but still active reaction centers to the decline of the J cannot be excluded.

Dissipation mechanisms

Light that can no longer be used for photosynthesis is expected to be dissipated as fluorescence and heat. Photoacoustic spectroscopy allows the quantification of heat emission as a function of the severity of the photoinhibitory treatment. In Fig. 7.6a the relationship between heat emission after a photoinhibitory treatment and the corresponding F_v/F_m is plotted. From an F_v/F_m of 0.87 down to an F_v/F_m of about 0.70 heat emission increases. Below this value the amount of emitted heat declines again.

As mentioned in the protocol of Fig. 7.1, photosynthesis was brought in a steady state 15 min after the cessation of the photoinhibitory treatment. During the 700 s actinic light non-photochemical quenching (q_N) is induced. An irradiance of 320 $\mu mol.m^{-2}.s^{-1}$ is not expected to be photoinhibitory and the largest part of the induced nonphotochemical quenching is expected to be energy quenching (q_E). In Fig. 7.6b the fraction of q_N induced by the actinic light that can be attributed to q_E is plotted as a function of F_v/F_m. Down to an F_v/F_m of about 0.70 the q_E-fraction increases from about 40% to 70%, below that value the contribution of q_E to q_N decreases again.

If q_E is plotted as a function of F_v/F_m (Fig. 7.7a) it is found that q_E decreases drastically below an F_v/F_m of about 0.78 and declines to a value close to zero around an F_v/F_m of 0.55. However, as inactivated reaction centers do not emit variable fluorescence part of the q_E-decline can be attributed to inactivation of reaction centers. In Fig. 7.7b q_E
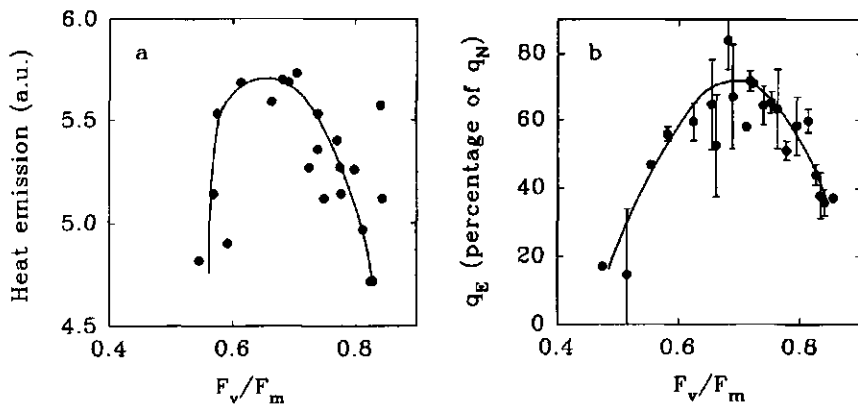
Fig. 7.6. Effect of a photoinhibitory treatment on the regulation of PSII. a. Relationship between heat release and the severity of the photoinhibitory treatment expressed as F_v/F_m. The heat release was defined as the level of heat release during the saturating pulse (Fig. 7.2a) b. percentage of q_N induced by the actinic irradiance attributable to q_E as a function of the severity of the treatment. The F_m and F_o-levels after the photoinhibitory treatment, just before turning on the actinic light were used as reference level for the calculation of q_N. The percentage of q_N recovered within 100s after turning off the actinic light was assigned to q_E. Bars represent standard deviation (n = 2-6).

is corrected for inactivated PSII reaction centers (see Materials and methods). A decline of q_E below an F_v/F_m of 0.78 is still seen. In Fig. 7.7c the relationship between the quenching related to photoinhibition (q_I) and q_E is plotted. Similarly as was found by Van Wijk and Van Hasselt (1993) a more or less linear relationship is found, even though in our case q_T was separated from q_E. However, there is a considerable deviation from linearity as q_I declines below 0.25. The induction of q_E is associated with F_o-quenching (Noctor *et al.* 1991). In Fig. 7.7d the ratio $(F_o-F_o')/F_o$ is plotted as a function of F_v/F_m. This ratio gives a measure for F_o-quenching. Under our conditions no F_o-quenching induced by the actinic illumination was found before F_v/F_m had dropped below 0.72-0.75. In a light response curve made with control leaves no or very little F_o-quenching was found in the range 0-800 μmol.m^{-2}.s^{-1} (not shown).

In Fig. 7.8a the amplitude of the q_I after a photoinhibitory treatment is plotted as a function of F_v/F_m and in Fig. 7.8b its halftime of relaxation assuming total recovery. For the halftime of relaxation the main effect is seen below an F_v/F_m of 0.70. Below an F_v/F_m-value of 0.70 a steep increase of the relaxation halftime is observed.
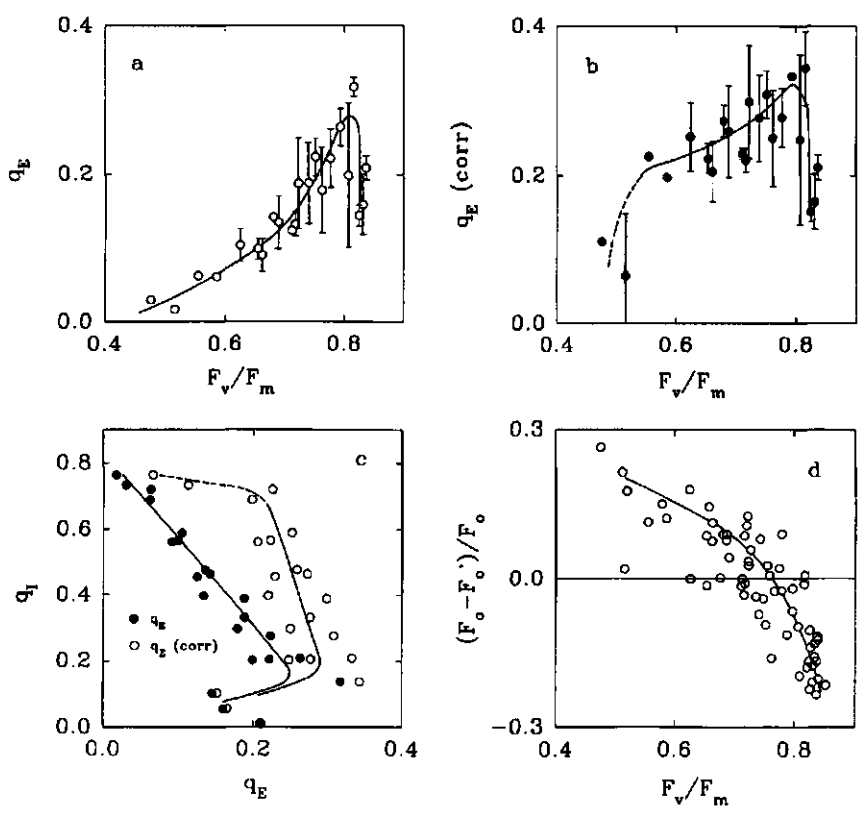
Fig. 7.7. Energy quenching (q_E) as a function of the severity of the photoinhibitory treatment expressed as F_v/F_m. The q_E is calculated relative to a reference F_m and F_o. a. The q_E normalized on F_o and F_m before the photoinhibitory treatment and b. q_E corrected for inactivated PSII reaction centers by using F_o and F_m measured after the photoinihibitory treatment. c. Relationship between the amplitude of q_I and q_E modulated by the severity of a photoinhibitory treatment. d. Relationship between F_o quenching induced by the actinic irradiance and the severity of the photoinhibitory treatment. Bars represent standard deviation (n = 2-6).

Discussion

F_v/F_m

As reviewed by Krause and Weis (1991) several studies have indicated that a linear relationship exists between F_v/F_m and the quantum yield of oxygen evolution and between F_v/F_m and the capacity of PSII for oxygen evolution. As can be seen in Fig. 7.3 in pea a linear relationship exists between the potential oxygen evolution rate of PSII and F_v/F_m. This indicates that in our studies F_v/F_m is also a good measure for the inactivation of PSII by the photoinhibitory treatment and can be used as an internal standard for the severity of a photoinhibitory treatment as advocated by several other authors (Adams *et al.* 1990, Ögren 1991 and Öquist *et al.* 1992a,b).

Inactive centers

Figure 7.3 also indicates that the inhibition of the potential oxygen evolution rate does not extrapolate to an F_v/F_m-value of 0 but to a value of around 0.44. In the study of Van Wijk and Krause (1991) with protoplasts of *Valerianella locusta* the relationship between electron transport and F_v/F_m extrapolated to an F_v/F_m-value of 0.40 and in the study of Krause *et al.* (1990) with spinach thylakoids to 0.42. These values are almost identical to the value we found. In several studies with leaves of various plant species the relationship between the quantum yield of oxygen evolution and F_v/F_m extrapolates to F_v/F_m-values higher than but close to zero (Demmig and Björkman 1987, Björkman *et al.* 1988, Tyystjärvi *et al.* 1989 and Adams *et al.* 1990). However, in the latter experiments oxygen evolution was measured using a leaf disc electrode. This method has the disadvantage that the whole leaf is assayed while only fluorescence emitted by the upper cell layers is detected. This means that inhibition gradients within the leaf (Krause and Weis 1991, see also Chapter 1) affect the results. Photoacoustic spectroscopy assays, like fluorescence, only the upper cell layers of the leaf (Buschmann 1989) and is therefore less sensitive to inhibition gradients. This methodological difference between photoacoustic spectroscopy on the one hand and leaf disc measurements on the other can explain the discrepancy between our data on intact leaves and those of others (Demmig and Björkman 1987, Björkman *et al.* 1988, Tyystjärvi *et al.* 1989 and Adams *et al.* 1990).

If the residual fluorescence at the F_v/F_m-level at which oxygen evolution ceased is ascribed to PSII reaction centers with an inactive oxygen evolution system (Giersch and Krause 1991 and Van Wijk and Krause 1991) it would mean that in the pea leaves we used, under our conditions less than 12% of the variable fluorescence can be ascribed to PSII-centers inactive in oxygen evolution.

Phosphorylation status of LHCII

Down to an F_v/F_m of 0.78 (mild photoinhibitory treatment) changes occur in leaves characterized by: 1. a small increase of F_o (not shown), 2. an increase of the fraction of

closed reaction centers and thus a decrease of q_P (Figs 7.4a and b), 3. an increase of q_E (Fig. 7.7a,b; amplitude) and of the fraction of q_N induced by the actinic irradiance that can be attributed to q_E (Fig. 7.6b; percentage) and 4. a decrease of the J (Fig. 7.5). There are several processes that might explain these phenomena. Realignment of chloroplasts in response to the photoinhibitory treatment (to minimize the absorption cross section) is unlikely as in that case a decrease of F_o would have been expected. Effects of connectivity between different antennas are expected to be small as even under optimal conditions (thylakoids inhibited for 50% by photoinhibition) the effects of connectivity have been evidenced to be small (Cleland *et al.* 1986, see also Chapter 2). The most likely explanation seems to be the phosphorylation state of LHCII. Photoinhibition causes an inhibition of the kinase (Schuster *et al.* 1986) resulting in dephosphorylation of LHCII. If the antenna size of PSII increases as a consequence of the dephosphorylation of LHCII, F_o is expected to increase.

Another phenomenon that was observed, was that the percentage of non-photo-chemical quenching that can be contributed to q_E increased from 40 to 70% as F_v/F_m declined from 0.84 to 0.70 (Fig. 7.6b). The increase of q_E (Fig. 7.7a,b) after a weak photoinhibitory treatment ($F_v/F_m > 0.75$) (Fig. 7.7) can also be explained by the reversal of protein phosphorylation during the photoinhibitory treatment which would cause q_T to decrease. It is a well known phenomenon that a photoinhibitory treatment causes dephosphorylation of the light harvesting complex (Schuster *et al.* 1986, Demmig *et al.* 1987, Cleland *et al.* 1990, Ebbert and Godde 1994). One of the causes of the dephosphorylation is the inactivation of the kinase (Schuster *et al.* 1986) and a higher activity of the phosphatase (Ebbert and Godde 1994). Fernyhough *et al.* (1984) showed a relationship between the pH of the lumen and phosphorylation, in which an increase of the lumen pH correlates to an increase in the phosphorylation.

Photochemical quenching
The parameter q_P is the ratio between variable fluorescence emitted by open PSII reaction centers and the total emitted variable fluorescence and contains information about the equilibrium between the rate of charge separation and the rate of electron transfer to PSI. In Fig. 7.4a q_P is plotted as a function of F_v/F_m. Initially there is a small decrease of q_P associated with the dephosphorylation of the antenna (see above). A larger antenna has a larger absorptive cross section and it is expected that more reaction centers close. From an F_v/F_m of 0.78 down to an F_v/F_m of 0.55 q_P is constant. There are several factors that influence the equilibrium: 1. the absorption cross section of the antenna, 2. the effect of pH and xanthophyll cycle associated quenching mechanisms on the fate of an exciton in open reaction centers (F_o-quenching), 3. the strength of the pull of PSI and 4. the presence of inactive centers that are expected to be closed continuously during the illumination and affect the calculation of q_P. Fig. 7.4b was used to correct q_P for inactive centers. However, more information can be extracted from Fig. 7.4b. Down to an F_v/F_m-

value of 0.70 the number of closed reaction centers increases considerably even though the total number of oxygen evolving centers declined by approximately 40% (Fig. 7.3). The redox state of Q_A depends on the equilibrium between the supply of excitons (excitation pressure) and the demand for electrons by PSI. Assuming that there is no drastic decrease of the demand for electrons the increase of the number of closed reaction centers can only be explained by a considerable increase of the excitation pressure. This points to an increased absorption cross section of the PSII antenna system and can be explained by dephosphorylation of the antenna system and possibly effects of connectivity between active and inactivated PSII reaction centers. Below a F_v/F_m of 0.70 the number of closed reaction centers starts to decline at a faster rate than the decline of the open reaction centers. A declining number of PSII reaction centers has to supply electrons to an unaltered number of PSI reaction centers. As a consequence more PSII reaction centers are in the open state. This shift in the reduction level of the PSII reaction centers should lead to an increased q_P but this does not happen. However, correcting q_P for inactive centers makes the predicted increase of q_P visible. The number of closed centers does not decline to zero; about 3.3% of the maximal variable fluorescence remains. This 3.3% deviates from the 10 to 12% found in Fig. 7.3. The explanation for this difference could be that the variable fluorescence emitted by the inactive centers is quenched by a low lumen pH just like active reaction centers. The number of open reaction centers declines to 0 close to an F_v/F_m-value of 0.44 (Fig. 7.4c) while the number of closed centers remains constant below an F_v/F_m-value of 0.60 (Fig. 7.4b). According to its definition (see above) q_P then declines to 0 (Fig. 7.4a, dashed line). A similar phenomenon has been observed by Öquist et al. (1992b, Fig. 6).

Heat emission
When measuring oxygen production photoacoustically heat release by the leaf is also measured. In Fig. 7.6a heat production is plotted as a function of F_v/F_m. The heat signal also contains a component attributable to PSI and this component is about 50% of the total heat-signal (Malkin et al. 1992, Fork and Herbert 1993). At F_v/F_m-levels down to 0.6 heat release increases by about 20%. This means that heat release by PSII can increase by up to 40%. As the severity of the photoinhibitory treatment increases, the number of active reaction centers declines. Inactivated centers do not emit variable fluorescence, and there are no indications that F_o of these reaction centers changes (Vass et al. 1993). Inactivated reaction centers have only a limited number of options to dissipate absorbed energy: emission as heat, transfer of excitons to antennas of still active reaction centers, and possibly the use of energy for chemical reactions. Eyletters and Lannoye (1992) showed that the increase in heat release is correlated with the formation of zeaxanthin. Below an F_v/F_m of 0.60 the heat release starts to decline again. This decline is difficult to explain. No large changes in F_o were observed. Therefore, an extensive decrease of the number of PSII reaction centers is unlikely. The only remaining

option seems to be the use of absorbed energy for chemical reactions like degradation of carotenoids and chlorophylls and lipid peroxidation.

Energy quenching
In Fig. 7.7a q_E was calculated using F_m and F_o before the photoinhibitory treatment for normalization. In this graph q_E starts to decline below an F_v/F_m of 0.78. Part of this decline can be explained by the availability of a smaller number of PSII reaction centers (only active reaction centers emit variable fluorescence). After correcting for the loss of active centers q_E still declines (Fig. 7.7b). This residual decline can be explained in two ways: 1. an increased proton permeability of the membrane which would cause an increased dissipation of the pH gradient over the thylakoid membrane, or 2. a higher pH in the lumen because of an inhibition of the electron transport rate (decrease of the excretion of protons in the lumen).

One can also, as done by Van Wijk and Van Hasselt (1993), plot the parameter q_I as a function of q_E. In this plot q_I is a measure for the fraction of inactivated centers and Fig. 7.7c indicates that there is a good linear correlation between the decline of q_E and the increase in q_I which again can be explained by a decline of the population of active PSII reaction centers. After correction for these inactivated centers (see above) Van Wijk and Van Hasselt (1993) observed that a correlation between q_E and q_I lacked. Our results (Fig. 7.7c) are at variance with those of Van Wijk and Van Hasselt (1993).

A second feature of Fig. 7.7a are the low q_E values of the non or negligibly photoinhibited leaves ($q_I \sim 0$). The most likely explanation for this phenomenon seems to be the presence of a considerable population of PSII reaction centers with a phosphorylated LHCII and thus a smaller antenna. Several studies have shown that LHCII is necessary for the expression of q_E (Lokstein *et al.* 1993 and Falk *et al.* 1994a,b) and if the outer antenna is smaller less q_E can be expected as there is less antenna available for the action of a low lumen pH. It is known that LHCII is dephosphorylated during a photoinhibitory treatment (see above) and this dephosphorylation process can explain very well the initial increase of q_E.

Photoinhibitory quenching (q_I)
In Fig. 7.8 two characteristics (amplitude and halftime) of q_I are illustrated. According to the fluorescence protocol (Fig. 7.1) the leaves were illuminated for 700 s with 320 μmol.m^{-2}.s^{-1}. During this period ATP is produced and maybe also D1-protein synthesized. Therefore, the recovery during the dark period after the 700 s light is not just a dark recovery. Recovery was only followed for about one hour and it is unclear if the recovery would be completed if the recovery time was indefinite. Comparing Fig. 7.8b with Fig. 7.5 shows that the decrease of the recovery rate (q_I) goes simultaneously with the decrease of the electron transport rate (J). The data in Fig. 7.8b indicate that the severity of the photoinhibitory treatment has a progressively increasing effect on the recovery

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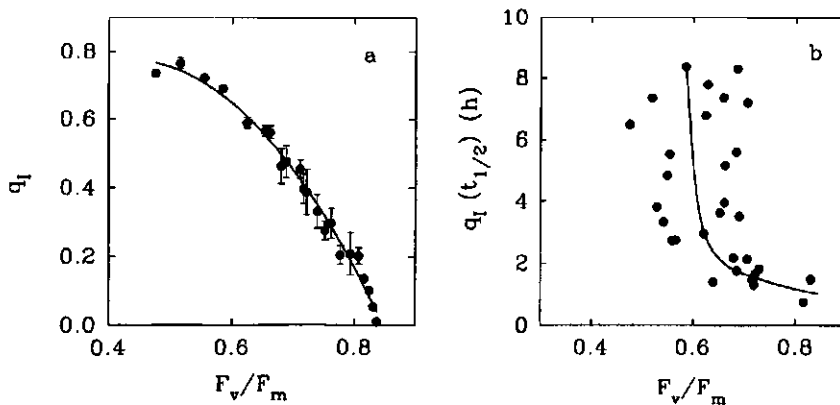
Fig. 7.8 Effect of a photoinhibitory treatment on q_I. The amplitude of q_I (a) and the halftime of relaxation of q_I (b) as a function of the severity of the photoinhibitory treatment expressed as F_v/F_m. The halftime of relaxation of q_I was derived from the relaxation kinetics of q_N in the dark (see Fig. 7.2). Bars represent standard deviation (n = 2-6).

time. The recovery rate of q_I (*i.e.* resynthesis of D1) depends on the production rate of ATP and possibly NADPH by whole chain electron transport. ATP is an important regulator of the translation rate of the D1 protein (Kuroda *et al.* 1992). After the photoinhibitory treatment PSI is known to be still active (Critchley 1981, Havaux and Eyletters 1991). Therefore, ATP synthesis by means of cyclic phosphorylation remains possible. The data in Fig. 7.8b indicate that cyclic electron flow is unable to compensate for the loss of ATP-production by whole chain electron transport.

A hypothetical interpretation of the data in Figs. 7.5 and 7.8b might be that as long as ATP is synthesized by whole chain electron transport at a rate comparable to the rate in uninhibited leaves, recovery processes can proceed at a rate comparable to that in uninhibited leaves. Once whole chain electron transport and ATP-production decline, the recovery rate declines too, leading to much longer halftimes of q_I-relaxation. When all reaction centers are inactivated no ATP can be formed anymore by whole chain electron transport and this means that the relaxation time of q_I will go to infinite values.

In conclusion, on the basis of the presented data it is possible to understand how electron transport reacts to a severe photoinhibitory treatment. Changes in the photosynthetic apparatus after no or a slight photoinhibitory treatment (F_v/F_m 0.78-0.85) are not entirely understood. It is suggested that these changes are caused by dephosphorylation of the antenna complex.

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8 General Discussion

In the last 10 years the study of photoinhibition has attracted much attention. Recent comprehensive reviews are those of Prasil *et al.* (1992) and of Aro *et al.* (1993). Consensus has been reached on some aspects of photoinhibition but others still remain controversial. Surprisingly, no consensus is reached yet on the primary mechanism of photoinhibition. Photosystem II has been such a central issue of photosynthesis research that the question if PSII limits electron transport under all conditions was often ignored. As discussed in Chapter 1 photoinhibition of PSII does not necessarily cause inhibition of overall photosynthesis. In this Chapter several aspects associated with photoinhibition and dealt with in the previous Chapters are discussed.

Primary site of inactivation by photoinhibition
In Chapter 5 the primary site of photoinhibition was studied in thylakoids of pea. In that study the primary lesion was found to be localized on the acceptor side of PSII. In cyanobacteria (Kirilovsky *et al.* 1988, 1990), in *Chlamydomonas* (Zer *et al.* 1994) and in spinach (Briantais *et al.* 1992) it was also reported that under *in vivo* conditions the primary lesion of photoinhibition was localized on the acceptor side of PSII. This validates our approach to use thylakoids for the study of photoinhibition. The data in Chapter 5 also show that the donor side of PSII is sensitive to photoinhibition though to a lesser extent. As discussed in Chapter 5 it is unclear if there exists a causal relationship between donor and acceptor side photoinhibition. The data do not allow a choice between a causal relationship and independence, although the difference in pH-dependence (Fig. 5.3) seems to favor two independent reactions.

Primary mechanism of photoinhibition; role of bicarbonate
Several mechanisms to explain the inactivation of the acceptor side of PSII have been postulated. Under anaerobic conditions it is possible to induce a double reduction of Q_A (Van Mieghem *et al.* 1989, Andersson *et al.* 1989, Styring *et al.* 1990, Van Wijk *et al.* 1992, Vass *et al.* 1992). Van Mieghem *et al.* 1989 hypothesized that double reduction of Q_A did also occur under aerobic conditions and that it was the first step leading to

photoinhibition. Several lines of experimental evidence indicate that this hypothesis is incorrect and that double reduction of Q_A does not occur under aerobic conditions. Using picosecond time-resolved fluorescence measurements Vass *et al.* (1993) were unable to observe the changes in fluorescence lifetimes associated with the double reduction of Q_A, while these changes were clearly observed in anaerobic samples. Double reduction of Q_A is only expected to occur in samples with a population of highly reduced PSII reaction centers. However, no correlation between the redox state of Q_A and the occurrence of photoinhibition was found (Ögren *et al.* 1991, Van Wijk and Van Hasselt 1993). In active reaction centers the repulsive effect of the negative charge on Q_A^- promotes the recombination reaction between Pheo$^-$ and P_{680}^+ and prevents the accumulation of reduced pheophytin. The double reduction theory postulates that double reduced Q_A will become protonated and will leave its binding site. Under these conditions the accumulation of reduced pheophytin is no longer prevented. Therefore, the recombination between P_{680}^+ and Pheo$^-$ should slow down in photoinhibited PSII reaction centers. Eckert *et al.* (1991) were unable to observe this effect. These three observations indicate that it is unlikely that double reduction of Q_A plays a role in the process of photoinhibition.

A second mechanism is the Q_B-site (Kyle *et al.* 1984, Ohad *et al.* 1990, Gong and Ohad 1991, Zer *et al.* 1994). The hypothesis of the group of Ohad is that the semiquinone under photoinhibitory conditions is able to damage PSII (Ohad *et al.* 1990). This hypothesis rests heavily on results with mutants in which the reoxidation rate of the plastoquinone pool was low. Although evidence was reported that the spectrum of the semiquinone corresponds well to the action spectrum of photoinactivation (Greenberg *et al.* 1989) this only explains UV-damage because the semiquinone does not absorb light above 450 nm (Greenberg *et al.* 1989).

A third mechanism is related to bicarbonate binding. Sundby (Sundby 1990, Sundby *et al.* 1992) tried to prove that bicarbonate release is the cause of the inactivation of PSII by studying if added bicarbonate could protect thylakoids against photoinhibition. We were unable to reproduce her results (not shown). The experimental setup of Sundby (1990) assumes that bicarbonate can easily rebind to PSII. As discussed in Chapter 3 there are several lines of evidence that make this assumption less likely. The non-competitive displacement of bicarbonate by NO and SiMo indicates that bicarbonate release might be an irreversible process and that the addition of bicarbonate can only give a transient protection against photoinhibition. Another approach is necessary to study if bicarbonate release is the reason for the inactivation of PSII. In Chapter 5, the pH-dependence of the inactivation of SiMo-mediated and FeCy-mediated electron transport was studied. Above pH 7 bicarbonate is bound very tightly to PSII (Stemler and Murphy 1983, Diner and Petrouleas 1990), while below pH 7 binding of bicarbonate to PSII becomes increasingly less tight. It is also known that the bicarbonate-effect is maximal around pH 6.5 (Vermaas and Van Rensen 1981). Ferricyanide-mediated electron transport is expected to be sensitive to bicarbonate release and SiMo-mediated electron transport is

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expected not to be sensitive to bicarbonate release. Fig. 5.3a indicates, that below pH 7 the sensitivity of FeCy-mediated electron transport to photoinhibition increases while the sensitivity of SiMo-mediated is hardly affected. The ratio between the halftime of inactivation of FeCy- and SiMo-mediated electron transport is maximal around pH 6.4 (Fig. 5.3b). Both phenomena are indicative of a bicarbonate effect. In Chapter 6, the problem was approached in a different way. Bicarbonate is thought to bind to the non-heme iron (Diner *et al.* 1991). Changes in the binding environment of the non-heme iron might influence bicarbonate binding. In Chapter 6 we made use of a site directed D2-mutant of *Synechocystis* 6803 (G215W) in which a glycine close to the non-heme iron was substituted by a tryptophan. This mutant was four times more sensitive to photoinhibition than the reference strain. One of the most significant differences between the mutant and the reference strain was that in the mutant electron transfer from Q_A to Q_B was 10 times more sensitive to formate inhibition than in the reference strain. These observations on the pH-dependence of the sensitivity of pea thylakoids to photoinhibition and on the cause of the higher sensitivity of a *Synechocystis* mutant to photoinhibition both support the hypothesis that there is a relation between photoinhibition and bicarbonate release. More experiments have to be devised to establish the relationship between photoinhibition and bicarbonate release more rigorously.

Amongst the three hypotheses explaining acceptor side inhibition we have a preference for the bicarbonate-release hypothesis. The bicarbonate binding site is located on the acceptor side of PSII, its occupation seems to be essential for effective electron transport and for a viable conformation of the protein (Chapter 3). In coordination with the non-heme iron the occupation of the site has a stabilizing effect on Q_A. Finally the pH profile of inactivation fits a bicarbonate effect (Chapter 5). In summary the loss of bicarbonate from PSII seems to be a viable explanation for the inactivation of PSII.

Photoinhibition is a dose-response effect
In several studies a linear relationship between the photon flux density and the rate of inactivation was observed. This indicates that the light energy dose determines the effect (Jones and Kok 1966, Ögren 1991, Wünschmann and Brand 1992, Tyystjärvi *et al.* 1994b, Nagy *et al.* 1995). The same dose-response effect was observed in Chapter 5 for pea thylakoids and in Chapter 6 for both cyanobacterial strains that were used. At first sight, a dose response relationship would not be expected if photoinhibition would be related to a photochemical reaction. For the Q_A-double reduction theory a dose response relationship means an increased probability that a reduced pheophytin will donate an electron to a reduced Q_A. In the semiquinone theory it means an increased probability that activated semiquinones will cause damage to PSII. For the bicarbonate release theory the relationship between dose and response is less clear. One explanation might be that under supersaturating light conditions the structure of PSII is affected, somewhat increasing the probability that a bicarbonate molecule escapes. For example charge separations are

thought to cause a contraction of the reaction center (Delosme *et al.* 1994) and maybe the shielding effect of LHCII on PSII (Jahns and Junge 1992) is influenced by the excitation level.

The study of the properties of bicarbonate bound to photosystem II

To prove that bicarbonate-release is the primary step leading to the inactivation of PSII it is necessary to find experimental tools to study the role of bicarbonate in PSII. Bicarbonate is an elusive molecule with properties that cannot easily be measured. Up till now bicarbonate was studied in two ways, the oldest method is exchanging bicarbonate for formate and to measure the changes in the properties of PSII. The other one is studying the system with EPR. Both methods are hampered by methodological problems. With respect to the first case it is valid to argue that the inhibitory effect of formate rather than the requirement for bicarbonate is studied. The second method requires low temperatures and sub-thylakoid particles. Thermoluminescence seems to be able to substitute partly for EPR (Demeter et al. 1994, Johnson *et al.* 1994) which would make it possible to work with leaves.

If photoinhibition is associated with or even is caused by release of bicarbonate (Chapter 3), photoinhibition could be used as a pretreatment to study other effects of bicarbonate. However, PSII inactivated by photoinhibition no longer displays variable fluorescence, is silent in terms of thermoluminescence and no longer evolves O_2. In other words all the tools that otherwise give us so much information on all kinds of functional parameters are all of a sudden no longer useful. Only SiMo is able to accept electrons under these circumstances. As shown in Chapter 4, SiMo a powerful tool to study the properties of bicarbonate in particular with respect to the binding properties of bicarbonate. However, it is an ineffective tool to get information on the physiological role of bicarbonate.

Another approach would be to explore modified PSII reaction centers. To study the effect of the outer antenna on the acceptor side of PSII, intermittent light grown plants seem a good option. Jahns and Junge (1992) have shown that in plants grown under these conditions the protonation rate of Q_B is affected. Principally the same phenomenon occurs in cyanobacteria where during the isolation of thylakoids the phycobilisomes are lost. In thylakoids of *Synechocystis* the reaction kinetics of SiMo differ strongly from the kinetics seen in pea thylakoids (not shown). An additional problem in thylakoids of cyanobacteria is the partial loss of the soluble electron carrier between cyt b_6/f and PS I (cytochrome c). Another way of modifying PSII is by making site directed mutants. In Chapter 6 a site directed mutant of *Synechocystis* PCC 6803 was characterized to find a correlation between altered bicarbonate binding properties of PSII in the mutant and an increased sensitivity to photoinhibition. The results indicate that site directed mutants can be a useful tool to study photoinhibition but only if the effects of the mutation on the properties of PSII can be characterized successfully.

Effect of photoinhibition on whole chain electron transport

In the previous sections possible mechanisms of PSII inactivation were discussed. Photoinhibition also affects whole chain electron transport. The plastoquinone pool serves several PSII reaction centers and inactivation of one PSII reaction center makes more plastoquinone molecules available to the remaining active PSII reaction centers. This means that depending on the site of the rate limitation there is no one to one relationship between inactivation of PSII and inactivation of electron transport. Electron transport is also affected by effects of photoinhibition on the formation and maintenance of the pH gradient. The lumen pH regulates antenna quenching (Horton and Ruban 1992, Dau 1994) and the oxidation rate of plastoquinol. The formation of a low lumen pH is dependent on PSII activity, but at the same time PSII activity is also inhibited indirectly by the formation of a low lumen pH (feedback regulation). A third parameter that determines the effect of photoinhibition on electron transport is the the rate at which chloroplasts can repair the damage done by photoinhibition. In the next sections several aspects of the effects of photoinhibition on photosynthetic electron transport are discussed.

Inactive centers

On the basis of spectroscopic heterogeneity of DCMU-poisoned chloroplasts the concept of PSII-reaction centers with different photosynthetic properties was introduced (Melis and Homann 1976). However, it is a matter of debate how many functionally distinguishable forms of PSII exist (Black *et al.* 1986).

In Fig. 7.3 we also identified a population of inactive centers. No attempt was made to determine to which category these inactive centers belonged. The properties which distinguished them from active reaction centers were their inability to produce oxygen under our conditions and their insensitivity to photoinhibition. About 10-12% of the variable chl *a* fluorescence could be ascribed to these inactive centers. Depending on their antenna size (up to twice as small as the antenna size of active centers (Thielen and Van Gorkom 1981)) this value could translate to as much as 20-25% of the total number of reaction centers. The F_v/F_m-value at which the oxygen evolution extrapolated to 0 corresponds well to F_v/F_m-values observed in comparable experiments with intact chloroplasts of spinach and protoplasts of *Locusta valerianella* (Krause *et al.* 1990, Van Wijk and Krause 1991). Knowledge of the fraction of inactive centers is important for the interpretation of the effects of photoinhibition on fluorescence parameters like the q_P and the ϕ_P (Chapter 7). The inactive PSII reaction centers mask an increase of the q_P in photoinhibited leaves (Fig. 7.4a).

Effect of photoinhibition on the rate limiting step of whole chain electron transport

The quantitative effect of photoinhibition on photosynthesis is different for different (partial) photosynthetic reactions. Determinations of PSII-activity and measurements of electron transport in the presence of an uncoupler and an electron acceptor show a good

correlation between the inactivation of PSII and the decrease of electron transport activity, independent of the irradiance at which the activity is determined (Bradbury and Baker 1986, Krause et al. 1990). In coupled chloroplasts and leaves the quantum yield for oxygen evolution was more affected than the maximal oxygen evolution rate (Ögren et al. 1984, Walker et al. 1986, Baker and Horton 1987, Björkman et al. 1988, Leverenz et al. 1990). In cyanobacterial cells (Chapter 6) and pea leaves (Chapter 7) that were not or only slightly photoinhibited, the effect of a rate limitation by a part of the electron transport chain other than PSII was observed (Figs. 6.9, 7.4b and 7.5). In Chapter 7 it was concluded that the rate limitation shifted from cyt b_6/f and the Calvin cycle to PSII as the inhibition of PSII progressed. It seems that the study of the effect of DCMU-inhibition on whole chain electron transport by Heber et al. (1988) is a good model to describe the effect of the inactivation of PSII by light on whole chain electron transport. Weinbaum et al. (1979) reported that in *Spirodela* a 85% depletion of the D1 protein resulted in only a 15% inhibition of the CO_2-fixation rate. These results are quite comparable to those found by Heber et al. (1988) for DCMU inhibition. These observations point to the conclusion that there is no simple relationship between photoinhibition of PSII and the inhibition of primary production.

ATP production and D1 synthesis
Inhibition of PSII is thought to be followed by degradation of the D1 protein. Recovery occurs by resynthesis of D1. In most studies it is assumed that the synthesis rate of D1 is independent of the severity of the photoinhibitory treatment. An effect of the irradiance during recovery on the rate of recovery has been shown (Aro et al. 1993, Sundby et al. 1993) and the recovery rate also seems to depend on the irradiance at which the plants are grown (Aro et al. 1993, Sundby et al. 1993). However, the relationship between the rate of D1 synthesis and the severity of the treatment has not been studied so far. This is rather surprising because as the fraction of inhibited PSII reaction centers increases, the synthesis of ATP will decrease and thus the ATP available for D1 synthesis will decrease. The experiments in Chapter 7 show a relation between the decrease of the photosynthetic flux (J) (Fig. 7.5) and the decrease in the recovery rate of photoinhibition (q_I) (Fig. 7.8b). Moreover, it was shown that the recovery time reaches infinite values as the number of active reaction centers declines to 0. Though this kind of experiments should be repeated at low irradiances during relaxation, the present results probably reflect a genuine decrease of the rate of D1 synthesis. The induction period was long enough to synthesize some D1, allowing some repair of PSII reaction centers even in darkness. It was shown that as long as the electron transport rate is not affected by the photoinhibitory treatment the recovery time of q_I is hardly affected. On the basis of these results it is recommended to study the relationship between the rate of D1 synthesis and the irradiance during the recovery period in leaves with variable fractions of active PSII reaction centers. Another important conclusion that can be drawn from the data in Fig. 7.8 is that

in pea leaves cyclic electron flow is unable to provide the ATP necessary for the repair of PSII.

Permeability of photosynthetic membranes to protons
Little information on the effect of a photoinhibitory treatment on the proton permeability of the thylakoid membrane is available. Any observed effect of photoinhibition on the proton permeability of the thylakoid membrane has to be corrected for effects on electron transport components. Whole chain electron transport (Critchley 1982, Ögren and Öquist 1984, Barenyi and Krause 1985, Bradbury and Baker 1986) to study permeability changes is unsuitable because whole chain electron transport is very sensitive to photoinhibition. The decrease of the ratio between whole chain electron transport in the absence and presence of an uncoupler (Ögren and Öquist 1984, Bradbury and Baker 1986) only shows that under the measuring conditions PSII becomes the limiting step as the length of the treatment increases and more PSII reaction centers become photoinhibited (compare Heber *et al.* 1988 and see above).

It is difficult to devise an experiment which makes it possible to evaluate the effect of a photoinhibitory treatment on the membrane permeability to protons of thylakoids. For results that are simple to interprete it is necessary to use a partial electron transport reaction sensitive to the lumen pH but insensitive to photoinhibition. Electron transport from $DCPIPH_2$/ascorbate to MV using thylakoids isolated from photoinhibited leaves seems a good system as it is almost insensitive to photoinhibition (Critchley 1982). We have chosen to study electron transport from $DCPIPH_2$/ascorbate to MV in photoinhibited thylakoids. This partial reaction is sensitive to photoinhibition in treated thylakoids but far less than whole chain electron transport. Our data show an increase of coupled electron transport after a photoinhibitory treatment (Chapter 5) which can be interpreted in terms of an increased proton permeability of the membrane. The data compare reasonably well with those reported by Tjus and Andersson (1993). Contrary to the data in thylakoids, in intact leaves very little indication for an increase of the proton permeability of the membrane was found (Chapter 7). The qE-values, corrected for PSII reaction centers inactivated by the photoinhibitory treatment, still declined somewhat. Other data indicated that a decrease of the number of protons excreted into the lumen could explain this decrease as well.

Control mechanisms and photoinhibition
One of the main conclusions of Chapter 7 is that still active PSII reaction centers are not affected by the photoinhibitory treatment. The efficiency of the still active PSII reaction centers increases as the pull from PS I and the Calvin cycle increases. In addition phosphorylation of LHCII is an important regulating mechanism in pea leaves. One of the reasons that no F_o-quenching was observed is probably that F_o-quenching by the transthylakoid pH was compensated for by a F_o-rise caused by dephosphorylation of LHCII. As

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documented in Fig. 7.4b initially the number of closed reaction centers increases as the severity of the photoinhibitory treatment increases. This indicates that the excitation pressure on the still active reaction centers becomes larger. A possible explanation for this increased excitation pressure is the larger antenna size after dephosphorylation, possibly in combination with connectivity between active and inactivated centers. However, little experimental evidence in the literature was found for a role of connectivity (see Chapter 2).

Our experiments do not allow conclusions about the involvement of the xanthophyll cycle in photoinhibition. Although the xanthophyll cycle may make PSII less sensitive to photoinhibition it had little effect on linear electron transport at moderate photon flux densities as used in our experiments.

Concluding remarks on the effects of photoinhibition on electron transport
The results in Chapter 7 show that the main effect of photoinhibition is the elimination of a part of the population of PSII reaction centers. Unfortunately our data do not allow a definite conclusion whether this elimination is exclusively due to the release of bicarbonate from the PSII binding site (Chapter 3), or not. All other effects are merely a consequence of the dwindling population of PSII reaction centers. As the number of PSII reaction centers declines the rate limitation shifts from cyt b_6/f and the Calvin cycle towards PSII which causes an increase of the population of oxidized PSII reaction centers. The residual decline of the q_E after correcting for PSII reaction centers inactivated by photoinhibition might indicate that the declining number of still active reaction centers is below a certain inhibition level no longer able to keep the lumen pH at the same level as in control leaves. The recovery data indicate that the rate of recovery depends on the rate of whole chain electron transport activity.

Summary

The photosynthetic apparatus is sensitive to excess light. This phenomenon is called photoinhibition. It affects specifically photosystem II (PSII) and is related in some way to the turnover of the D1 protein, a central component of PSII.

Measurements performed with plant systems that were photoinhibited under *in vivo* conditions give evidence for the conclusion that the photoinactivation site is localized on the acceptor side of PSII. Several mechanisms have been postulated to explain the inactivation process. In Chapter 3, one of these mechanisms is treated more extensively. The protonation of the secondary electron acceptor, Q_B, is as yet badly understood. It is hypothesized (Chapter 3) that the acceptor side of PSII shows carbonic anhydrase activity. A CO_2 and a H_2O molecule can bind to the non-heme iron and can react with each other to form bicarbonate and to release a proton. The theory postulates that during a photoinhibitory treatment the probability that bicarbonate/CO_2 disappears from its binding site increases. It is further argued that this loss is irreversible.

Silicomolybdate is an electron acceptor that is able to accept electrons from the non-heme iron. Binding of SiMo to its acceptor site causes displacement of bicarbonate/CO_2. In Chapter 4 the interaction between SiMo, bicarbonate/CO_2 and PSII was analyzed. Information was obtained on the binding site of SiMo, and the binding characteristics of both SiMo and bicarbonate/CO_2. The characterization of SiMo binding was necessary to be able to use the compound for photoinhibition studies.

In Chapter 5 it was established that in pea thylakoids the inactivation site of photoinhibition is indeed located on the acceptor side of PSII. Further it was observed that the donor side is also inactivated though at a much slower rate. Photoinactivation of both donor and acceptor side are light dose dependent. Displacement studies of bicarbonate/CO_2 with nitric oxide (NO) and SiMo indicated that the displacement of bicarbonate is irreversible. As expected, the addition of bicarbonate does not give any lasting protection against photoinhibition. The pH-dependence of acceptor side inactivation corresponds with theoretical considerations of bicarbonate/CO_2 behavior: an increased sensitivity towards photoinhibition below pH 7 and a maximum difference between the rate of donor and acceptor side inactivation around pH 6.4. These observations support the theory that bicarbonate release is responsible for the photoinactivation of PSII.

In Chapter 6 a site-directed mutant of *Synechocystis* sp. PCC 6803 was used to find support for our hypothesis. This mutant is mutated in the binding environment of the non-heme iron. It is four times more sensitive to photoinhibition than a reference strain. One of the main effects of the mutation is a ten times higher sensitivity to formate (formate displaces bicarbonate). This indicates that bicarbonate is more loosely bound to PSII in this mutant. This may explain the increased sensitivity to photoinhibition and in that case this result supports our hypothesis.

In Chapter 7 the effects of photoinhibition on the regulation of photosynthetic electron transport were studied. A combination of photoacoustic and fluorescence spectroscopy was used. A small population of PSII reaction centers was found that does not produce oxygen, but does fluoresce. The fluorescence data were corrected for these inactive centers. Initially, the fraction of reduced PSII reaction centers increases as a consequence of the photoinhibitory treatment (photochemical quenching, q_P, decreases). Possibly this change is brought about by dephosphorylation of the antenna complex. A more severe photoinhibitory treatment causes an oxidation of the PSII reaction centers (q_P increases). Other components of the electron transport chain, apart from PSII, are hardly affected by a photoinhibitory treatment and therefore, the demand for electrons remains at the same level. As a consequence the still active PSII reaction centers can work progressively more efficient. The decline of energetic quenching, q_E, during a photoinhibitory treatment could almost entirely be explained by a decline of the available number of PSII reaction centers. A small part of the decline of q_E has other causes, possibly an increase of the lumen pH as a consequence of a lower proton excretion into the lumen or an increased proton permeability of the thylakoid membrane. The fluorescence data also indicated that the recovery rate of photoinhibition depends on the rate of ATP synthesized by linear electron transport. Cyclic electron transport is not able to compensate for the lost capacity of linear electron transport to induce ATP synthesis during a photoinhibitory treatment.

In conclusion, the effects of a moderate photoinhibitory treatment in pea leaves can be explained by dephosphorylation of the antenna system. The effects of a severe photoinhibitory treatment are caused by a progressive inactivation of PSII. Indications were collected supporting the hypothesis that bicarbonate/CO_2 release is the trigger leading to the inactivation of PSII.

Samenvatting

Het fotosynthesesysteem van planten is in staat licht om te zetten in chemische energie (bijvoorbeeld suikers). In de chloroplast zijn een tweetal eiwitcomplexen (de fotosystemen I en II) aanwezig, die in staat zijn om licht te absorberen en te converteren naar een elektronen- en protonenstroom. Fotosysteem II (PSII) blijkt geïnactiveerd te kunnen worden door licht. Dit proces wordt fotoinhibitie genoemd. Fotosysteem II bestaat uit een groot aantal eiwitten, maar slechts één van deze eiwitten, het D1 eiwit, wordt als een gevolg van de inactivatie van PSII afgebroken.

Dit proefschrift richt zich op een tweetal aspecten van fotoinhibitie: 1. het mechanisme waarmee licht PSII inactiveert, en 2. de wijze waarop de inactivatie van PSII het fotosynthetisch electronentransport beïnvloedt.

Het reactiecentrum van PSII bestaat aan de donor zijde uit een watersplitsend systeem, dat in staat is electronen te doneren aan een chlorofyl molekuul (P_{680}). Excitonen uit de antenne zijn in staat de overdracht van een elektron van P_{680} naar een feofytine molekuul aan de andere kant van de membraan te induceren (ladingsscheiding). Deze ladingsscheiding wordt min of meer onomkeerbaar gemaakt door overdracht van het elektron aan een chinon molekuul (Q_A). Dit kan het elektron overdragen aan een tweede chinon (Q_B) dat na opname van twee elektronen en twee protonen PSII kan verlaten. Hoe de protonatie van Q_B in zijn werk gaat is nog steeds onduidelijk. Een bicarbonaat/CO_2 molekuul en een non-haem ijzer, die zich tussen Q_A en Q_B bevinden, zouden hierbij een rol kunnen spelen.

Metingen aan systemen, die onder *in vivo* condities zijn gefotoinhibeerd, localiseren het lichtgeïnduceerde defect aan de acceptor zijde van PSII. In de literatuur zijn een drietal mechanismen naar voren gebracht om deze inactivatie te verklaren. In Hoofdstuk 3 is één van deze mechanismen verder uitgewerkt. Er is van uit gegaan, dat de acceptor zijde van PSII carbonzuur anhydrase activiteit vertoont. Dit betekent, dat een CO_2 en een H_2O molekuul worden omgezet in een bicarbonaat molekuul en een proton. Dit proces wordt versneld door het katalyserende effect van het non-haem ijzer. Het proton is beschikbaar voor de protonering van Q_B^-. Verder werd gepostuleerd, dat als gevolg van fotoinhibitie het bicarbonaat/CO_2 molekuul PSII verlaat en dat dit een irreversibel proces is. Hierdoor wordt de protonatie van het Q_B-molekuul vrijwel onmogelijk gemaakt en

wordt PSII geïnactiveerd.

Silicomolybdaat (SiMo) is een elektronenacceptor die waarschijnlijk in staat is om elektronen te accepteren van het non-haem ijzer. Als gevolg van SiMo-binding wordt bicarbonaat/CO_2 verdrongen. In Hoofdstuk 4 is de interaktie tussen SiMo, bicarbonaat/CO_2 en PSII gekarakteriseerd en werd informatie verkregen over bicarbonaat/CO_2 binding aan PSII. Tevens was de karakterisering van SiMo nodig om deze verbinding te kunnen gebruiken voor fotoinhibitie experimenten.

In Hoofdstuk 5 is gebruik gemaakt van de verschillen in bindings en elektronenacceptor eigenschappen van SiMo en FeCy (ferricyanide) om het fotoinhibitie proces te kunnen bestuderen. In thylakoiden van erwt blijkt de fotoinactiveringsplaats ook gesitueerd te zijn aan de acceptorzijde van PSII. De donorzijde van PSII wordt eveneens geïnactiveerd, maar dit proces verloopt aanzienlijk langzamer dan acceptorzijde inactivatie. Zowel donor- als acceptorzijde inactivatie zijn afhankelijk van de lichtdosis. Omdat verlies van bicarbonaat/CO_2 vermoedelijk een irreversibel proces is kan door toevoeging van extra bicarbonaat geen bescherming tegen fotoinhibitie worden verkregen. Uit de literatuur komt naar voren dat de binding van bicarbonaat/CO_2 pH-afhankelijk is. De pH-afhankelijkheid van de acceptorzijde inactivatie snelheid is zoals op basis van de bindings-eigenschappen van bicarbonaat/CO_2 aan PSII zou worden verwacht: een toename van de inactivatie snelheid beneden pH 7 en een maximaal verschil tussen de inactivatiesnelheid van acceptorzijde en donorzijde rond pH 6.4. Deze observaties ondersteunen de hypothese dat fotoinactivatie van PSII veroorzaakt wordt door verlies van bicarbonaat/CO_2.

In Hoofdstuk 6 is geprobeerd ondersteuning te vinden voor de hypothese met behulp van een mutant van de cyanobacterie *Synechocystis* sp. PCC 6803 die gemuteerd is in de bindingsomgeving van het non-haem ijzer. Deze mutant is vier keer zo gevoelig voor fotoinhibitie als een referentiestam. Eén van de belangrijkste consequenties van de mutatie is, dat de mutant tien keer gevoeliger is voor formiaat-binding (formiaat verdringt bicarbonaat) dan de referentiestam. Dit wijst op een lossere binding van het bicarbonaat/CO_2 molekuul aan PSII. Deze lossere binding zou de verhoogde gevoeligheid van de mutant voor fotoinhibitie kunnen verklaren en ondersteunt in dat geval de hypothese.

In Hoofdstuk 7 is aandacht geschonken aan effecten van fotoinhibitie op de regulatie van het elektronentransport. Dit werd gedaan met behulp van een combinatie van fotoakoestische en fluorescentie spectroscopie. Er werd een kleine populatie PSII reactiecenters gevonden die geen zuurstof produceren, maar wel fluorescentie uitzenden. De fluorescentie data werden voor deze populatie gecorrigeerd. Dit leidde tot het volgende beeld: In eerste instantie neemt door de fotoinhibitie behandeling de fractie gereduceerde PSII reactie centra toe (de fotochemische doving, q_P, neemt af). Mogelijk wordt dit veroorzaakt door defosforylatie van het antenne complex. Een zwaardere fotoinhibitie behandeling leidt tot oxidatie van de populatie niet geïnactiveerde PSII reactiecentra (de q_P neemt toe). De rest van het fotosynthesesysteem wordt niet beschadigd door de fotoinhibitie behandeling en de vraag naar elektronen van PSII verandert dus

ook niet. Dit betekent dat de nog actieve PSII reactie centra steeds efficiënter kunnen gaan werken.

De afname van de energetische doving, q_E, als gevolg van een fotoinhibitie behandeling kan vrijwel geheel verklaard worden door afname van de beschikbare PSII reactiecentra. Een klein deel van de afname moet worden toegeschreven aan een toename van de lumen pH (lagere proton excretie in het lumen of een toename van de protonpermeabiliteit van het thylakoidmembraan). Verder werden aanwijzingen gevonden dat de herstelsnelheid van fotoinhibitie-schade bepaald wordt door de beschikbaarheid van ATP uit lineair elektronentransport. Cyclisch elektronentransport kan niet in deze behoefte voorzien.

Samenvattend, de veranderingen in een erwteblaadje na een lichte fotoinhibitie behandeling kunnen verklaard worden door dephosphorylatie van het antennesysteem. Het effect van een zware fotoinhibitie behandeling op het functioneren van het fotosynthese systeem van erwteblaadjes wordt veroorzaakt door een progressieve inactivering van PSII. Er zijn aanwijzingen gevonden, die de hypothese ondersteunen, dat de inactivering van PSII door licht veroorzaakt wordt door verlies van bicarbonaat/CO_2.

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Curriculum vitae

Gert Schansker werd geboren op 16 mei 1966 te Loppersum. Na het behalen van het VWO diploma aan het Ommelander College te Appingedam, begon hij in 1984 met de studie planteziektenkunde aan de toenmalige Landbouwhogeschool in Wageningen. De doctoraalfase van de studie bestond uit twee hoofdvakken: fytopathologie (bij dr.ir. M.A. de Waard) en nematologie (bij drs. R. Dorhout en dr. F.J. Gommers) en een bijvak plantenfysiologie (bij dr. J.J.S. van Rensen). Tussendoor werden drie maanden doorge-bracht op een onderzoekslaboratorium van Dow Chemical in Letcombe Regis (UK). Het doctoraalexamen werd in januari 1990 met lof behaald. In Oktober van dat jaar is hij begonnen als onderzoeker in opleiding (OIO) voor de Stichting voor Scheikundig Onderzoek in Nederland gefinancierd door de Nederlandse Organisatie voor Wetenschap-pelijk Onderzoek (SON-NWO) bij de toenmalige vakgroep Plantenfysiologisch Onderzoek van de Landbouwuniversiteit te Wageningen. Het onderzoek aan deze vakgroep heeft geleid tot dit proefschrift. Tijdens het promotie onderzoek werd een periode van 6 maanden doorgebracht aan Arizona State University te Tempe in de Verenigde Staten. Sinds 1 oktober 1995 verricht hij onderzoek naar de donor zijde van fotosysteem II aan het Institute of Materials Science NCSR Democritos te Athene.