Molecular aspects of herbicide binding in chloroplasts

Molekulaire aspekten van herbicide binding in chloroplasten

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Molecular aspects of herbicide binding in chloroplasts

Proefschrift
ter verkrijging van de graad van
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op gezag van de rector magnificus,
dr. H. C. van der Plas,
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There is nothing more painful
than the insult to human dignity,
nothing more humiliating than servitude.
Human dignity and freedom are our birthright.
Let us then defend them or die with dignity.

Cicero.

STELLINGEN

1. Het model van Kok et al. ter verklaring van de waargenomen oscillaties in flitsgeïnduceerde zuurstofproduktie in geïsoleerde thylakoiden verklaart de gebeurtenissen meer op empirische dan op theoretische gronden, en voldoet mede daardoor niet wanneer een nauwkeurige simulatie van deze oscillaties verlangd wordt.

Kok B., Forbush B. and McGloin M. (1970) Cooperation of charges in photosynthetic oxygen evolution. I. A linear four-step mechanism. Photochem. Photobiol. 11, 457-475.

Dit proefschrift.

2. De veronderstelling dat na langdurige donkeradaptatie alle fotosystemen II in eenzelfde redoxtoestand verkeren is een onjuiste generalisering van de werkelijkheid, maar desalniettemin een vaak noodzakelijke aanname bij het verklaren van waargenomen verschijnselen.

Dit proefschrift.

3. De waarneming van Carpentier et al. dat het elektronentransport in triazine-resistente planten toch geremd kan worden met zeer hoge atrazin-concentraties, hoeft niet te wijzen op een additionele herbicide-bindingsplaats, maar kan ook verklaard worden uit een andere herbicide-bindingskinetiek.

Carpentier R., Fuerst E.P., Nakatani H.Y. and Arntzen C.J. (1985) A second site for herbicide action in photosystem II. Biochim. Biophys. Acta 808, 293-299.

Dit proefschrift.

4. In tegenstelling tot een door Pfister en Arntzen gesuggereerde verklaring voor triazine-resistentie, waarbij verminderde binding aan het D1 eiwit werd verondersteld, is eerder een sterk versneld loslaten van het herbicide van de bindingsomgeving verantwoordelijk voor de resistentie.

Pfister K. and Arntzen C.J. (1979) The mode of action of photosystem II specific inhibitors in herbicide resistant weed biotypes. Z. Naturforsch. 34c, 996-1009.

Dit proefschrift.

5. De mate van resistentie van planten tegen de werking van herbiciden kan bij gebruik van verschillende meetmethoden zo sterk uiteenlopen, dat het aanbeveling verdient onderscheid te maken tussen bijvoorbeeld *in vivo* en *in vitro* waarden voor de I_{50}-concentraties.

Van Oorschot J.L.P. and Van Leeuwen P.H. (1987) Inhibition of photosynthesis in intact plants of biotypes resistant or susceptible to atrazine and cross-resistance to other herbicides. Weed Res. 28, 223-230.

6. Fotosysteem II herbiciden als ureum-verbindingen, triazinen, fenolen en cyanoacrylaten verhinderen de Q_B binding competitief, en niet door allostere interakties.

7. De bijna onbegrensde mogelijkheden die de nieuw(st)e gen-amplificatie technieken bieden, vereisen bij iedere toepassing ervan een ethische verantwoording van de onderzoeker.

8. Het introduceren van een nieuw model ter verklaring van een bekend verschijnsel stuit bij veel onderzoekers vaak op een grotere weerstand dan op basis van wetenschappelijke benadering zou mogen worden verwacht.

9. De behoefte aan geld en publiciteit (ver)leidt wetenschappers steeds vaker tot het beschrijven van opzienbare maar helaas voor meerdere interpretaties vatbare resultaten.

10. Zodra de wetenschap een verband poogt te leggen tussen geest en lichaam ontstaat er bij velen een onverklaarbare en onredelijke angst voor het onbekende.

11. Het koopgedrag van de nederlandse konsument is door ondeskundigheid in kombinatie met misleidende of onvolledige voorlichting uiterst wispelturig en inkonsekwent geworden.

12. De manier waarop Kunst en Kultuur ondersteund worden staat in schril kontrast tot de algemene waardering ervoor.

Stellingen behorende bij het proefschrift "Molecular aspects of herbicide binding in chloroplasts" van Dirk Naber, Wageningen, 27 oktober 1989.

Voorwoord

Vanaf deze plaats, voor velen het eerst opengeslagen deel van een proefschrift, wil ik iedereen bedanken die op enigerlei wijze een bijdrage heeft geleverd aan de totstandkoming hiervan. Een aantal mensen is echter zo nauw bij mijn werk op de vakgroep betrokken geweest, dat ik hen graag apart wil noemen.

In de eerste plaats wil ik mijn co-promotor, dr. van Rensen, bedanken voor alle energie die hij de afgelopen jaren in het begeleiden van mijn onderzoek heeft gestoken. Beste Jacques, jouw rustige en gedegen aanpak hebben me erg geholpen bij de uitvoering van mijn onderzoek en de verwerking ervan tot dit proefschrift. Ook de samenwerking met de promotor, prof. Vredenberg, dient hier vermeld te worden. Beste Wim, de kritische manier waarop je steeds de resultaten bekeek dwongen me tot een nauwkeurige opzet van het onderzoek en verwerking van de resultaten ervan.

Jan Hobé heeft met grote nauwgezetheid veel experimenteel werk uitgevoerd. Jan, bedankt voor de prettige samenwerking en de inzet waarmee je je in de laatste jaren voor je welverdiende pensioen nog op een voor jou nieuw werkterrein hebt begeven.

Jaap Ooms, Willem Buurmeijer, Jan Snel en Olaf van Kooten wil ik graag bedanken voor de wijze waarop zij allen mij geholpen hebben het onderzoek te maken tot wat het is geworden; voor de discussies die we hadden, de praktische hulp, en de manier waarop jullie hebben getracht mij enig benul van fotosynthese, computers en programmeren bij te brengen. Mede door jullie enthousiasme en soms relativering is het voor mij een leuke en vruchtbare tijd geweest.

Eric Ivens bedank ik voor de inzet waarmee hij zich gedurende de korte tijd van zijn doctoraalvak met het onderzoek heeft beziggehouden.

Willem Tonk en Rienk Bouma ben ik veel dank verschuldigd, omdat zij met de ontwikkeling van de Joliot-electrode een zo belangrijke bijdrage hebben geleverd, dat zonder hun inspanningen mijn onderzoek niet

uitgevoerd had kunnen worden. Het tuinpersoneel wil ik bedanken voor het kweken en verzorgen van alle planten, die ik vervolgens fijngemalen heb.

Mein herzlicher Dank gilt auch Prof. A. Trebst und Dr. Udo Johanningmeier von der Ruhr-Universität Bochum in Deutschland. Sie ermöglichten mir, einige wertvolle Experimente am Lehrstuhl Biochemie der Pflanzen erfolgreich durchzuführen. Praktische Unterstützung bei meinem Aufenthalt in diesem Department erhielt ich von Ursula Altenfeld und Ulrich Bodner.

I thank Dr. John Phillips for the gift of the non-commercial cyanoacrylate herbicides, which were an essential part of my research.

En tenslotte: lieve Elly, bedankt voor alles!

Contents

Abbreviations and symbols

α_n	miss parameter for S_n to S_{n+1} transition
β	double hit parameter
a.u.	arbitrary units
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
ATPase	thylakoid membrane H^+-ATP synthetase complex
atrazine	2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine
CTAB	cetyl-trimethyl-ammonium bromide
cDNA	complementary DNA strand
cpm	counts per minute
ctDNA	chloroplast DNA
cyt	cytochrome
D1	PS II reaction center protein, carrying acceptor Q_B
D2	PS II reaction center protein, carrying acceptor Q_A
dATP	deoxy adenosine triphosphate
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
dCTP	deoxy cytidine triphosphate
ddNTP	dideoxy nucleoside triphosphate
dGTP	deoxy guanosine triphosphate
DNA	deoxy ribonucleic acid
DNAse	DNA digesting enzyme
dNTP	deoxy nucleoside triphosphate
DS	denaturation solution
DTT	dithiothreitol
dTTP	deoxy thymidine triphosphate
EB	extraction buffer
EDTA	ethylene diamino tetra acetate
EtBr	2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide
Fd	ferredoxin
FeCy	potassium ferrihexacyanate
FeS	Rieske iron-sulfur protein
IRM	isolation and reaction medium
kb	10^3 base pairs
kD	10^3 Dalton
L	light subunit of bacterial PS II reaction center
mRNA	messenger RNA
MW	molecular weight
$NADP^+$	nicotinamide-adeninedinucleotide (oxidized)
NADPH	nicotinamide-adeninedinucleotide (reduced)
NaAc	sodium acetate

NS	neutralization solution
OEC	oxygen evolving complex
P680	photosystem II reaction center
P700	photosystem I reaction center
PAA	polyacrylamide
PB1, PB2	precipitation buffers 1 and 2
Pheo	pheophytin
PQ	plastoquinone
PQH_2	plastoquinol
PS I	photosystem I
PS II	photosystem II
Q_A	primary quinone electron acceptor of photosystem II
Q_B	oxidized secondary quinone electron acceptor
Q_BH_2	reduced secondary electron acceptor (plastoquinol)
R	triazine-resistant biotype
RC	reaction center of photosystem II
RNA	ribonucleic acid
RNAse	RNA digesting enzyme
rRNA	ribosomal RNA
S	herbicide sensitive biotype (wildtype)
SM	shock medium
S_n	oxidation state of the oxygen evolving complex
ss	single stranded
SSC	standard sodium citrate
TE	Tris-EDTA solution
Tricine	N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine
Tris	tris(hydroxymethyl)aminomethane
u	unit(s) enzyme

1 General introduction

1.1 Introduction

A high crop yield cannot be obtained without crop protection measures. In Western agriculture crops are nowadays protected from losses caused by weeds mainly by chemical control using herbicides. At present it becomes more and more evident that the amount of chemicals to be spread in the environment must be kept as low as possible. Several measures to achieve this goal, e.g. crop rotation or temporary inundation, have been introduced into practice already, several others are under investigation. One way to decrease the amount of chemicals brought in the environment is to apply more active pesticides. A possible way to achieve this can be, in some cases, application of only one stereo-isomer of a compound containing a chiral carbon atom instead of a racemic mixture. Especially in the classes of plant growth regulating compounds often only one of the isomers shows appreciable activity (for a review see Naber and Van Rensen 1988).

This study was undertaken to obtain more knowledge about interactions of herbicides with their binding environment on the structural and molecular level. This information may help to design more active and for the environment possibly less dangerous herbicides in the future.

1.2 Plant photosynthesis

Photosynthesis in green plants, algae and photosynthetic bacteria is by far the most important energy-conserving process on earth. Light is absorbed through the plant by a wide variety of pigment-protein complexes, which enable the plant to use light of almost all visible colors

except green. The light energy induces a complex and highly organized electron transport. Via a chain of redox reactions the energy is used directly to reduce $NADP^+$ to NADPH, and indirectly to phosphorylate ADP to ATP. The energy is thus stored in the form of reducing equivalents (NADPH) and high-energetic molecules (ATP). These compounds can be used at sites where redox or chemical energy is needed. During its life-cycle the plant stores the energy in the form of long-living chemical bonds, mainly through the fixation and reduction of CO_2 to various sugar compounds and starch. In this form the radiation energy from the sun is preserved, until after the death of the plant microbiological organisms reduce the plant material further and turn them into fossil fuels.

1.3 The chloroplasts of higher plants

Plant cells have special organelles in which photosynthesis takes place: the chloroplasts. The internal chloroplast stroma is separated from the cell by a double membrane, the chloroplast envelope. Within the stroma an extensive and continuous membrane system, the thylakoid membrane, encloses a lumen. The thylakoid membrane accommodates all protein complexes, pigments and electron-carriers which are directly involved in the primary photosynthetic processes. In the chloroplast stroma the proteins performing the so-called photosynthetic dark-reactions are found. These are the CO_2-fixation reactions which, in contrast to the electron transport, can proceed in the dark using NADPH and ATP.

1.3.1 The chloroplast genome

Chloroplasts contain large, circular DNA molecules. The ctDNA has a size of about 120-155 kb, and codes for many chloroplast proteins. The complete DNA sequence was determined in *Marchantia polymorpha* (Ohyama et al. 1986) and *Nicotiana tabacum* (Shinozaki et al. 1988). About 55 different proteins are found in thylakoid membranes. The genes coding

for 49 of these proteins have been identified, and about half of these are located on the ctDNA (Herrmann, pers. comm.). The chloroplast DNA is coding for the reaction center (RC) proteins of PS I and II, most cytochromes and the catalytic subunits from the ATPase complex.

The regulation of chloroplast protein synthesis and the protein processing require very complicated mechanisms. Each chloroplast contains 10 to several hundred copies of the DNA, and each plant cell encloses many chloroplasts. Thus a very strict stoichiometric regulation of transcription and translation of the genes coding the chloroplast proteins is required, since the nuclear genes usually occur as single copies. For further reading see Ellis (1977), Chua and Gillham (1977) and Harwood and Walton (1988).

1.3.2 The electron transport chain in chloroplasts

The thylakoid membrane has a high protein content (about 70 %) as compared with most other biological membranes (Guidotto 1972). This indicates a high enzymatic activity. In total, about 55 different proteins are embedded in or closely associated with the membrane. Four large amino acid complexes are found: the two photosystems I and II, a cytochrome complex, and the ADP-phosphorylating (ATPase) complex. Three of the thylakoid membrane complexes are involved in electron transport: PS I, PS II and cyt b_6/f. For reviews on structure and function of the photosystems I and II the reader is referred to Van Gorkom (1985), Ghanotakis and Yocum (1985), Barber (1987), Glazer and Melis (1987) and Andréasson and Vänngård (1988). Associated with PS II is the oxygen evolving complex (OEC). This complex can accumulate four oxidizing equivalents used for splitting two molecules of water into protons, electrons and O_2. For reviews on the water splitting complex see Amesz (1983), Andersson et al. 1985, Dismukes (1986 and 1988) and Renger (1987). The cytochrome b_6/f complex is reviewed by Hauska (1985). The orientation of the protein complexes is schematically drawn in Fig. 1.1.

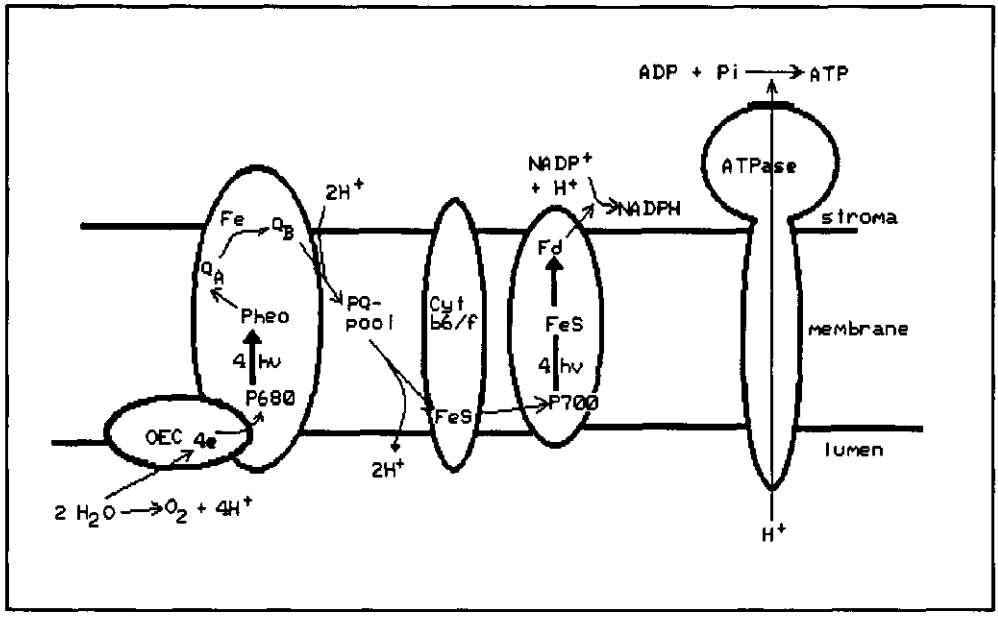


Fig. 1.1. The major membrane complexes involved in photosynthetic electron transport. Arrows indicate the electron transport path; bold arrows are the light-requiring steps.

Light energy is absorbed by the light harvesting pigment-protein complexes, associated with both photosystems, or directly by the antenna pigments. A chlorophyll *a* dimer, indicated according to its absorption maximum with P_{680}, is bound to the PS II reaction center. Upon illumination this so-called special pair is excited within 100 ps via the closely associated antenna chlorophyll-protein complexes (Hunter et al. 1989). A stable charge separation results in an oxidized P^+_{680} and, via an intermediary pheophytin, in a reduced primary quinone acceptor Q_A^-. The electron which is extracted from the reaction center is supplied from the water oxidizing side of PS II. The first molecule to be oxidized by P^+_{680} is the primary donor Z, recently identified as a tyrosine residue of the D2 protein (Barry and Babcock 1987, Debus et al. 1988). This donor in its turn is able to extract an electron from the OEC. This complex, consisting of

3 polypeptides, is located at the inner side of the thylakoid membrane, and it contains 4 Mn-atoms which serve as the actual electron donors. The manganese ions are probably in close interaction with a histidine residue from the D1 protein (Tamura et al. 1989). When 4 oxidizing equivalents are accumulated by the complex, two molecules of H_2O can be oxidized to form O_2 and 4 H^+ (Kok et al. 1970). The different redox states of the OEC, indicated as the S-states, are stable for at least several seconds. At the acceptor side the electrons are transported via the one-electron acceptor Q_A to the secondary acceptor Q_B, which are both plastoquinone molecules. Q_B can accept 2 electrons, and takes up 2 protons from the stroma. It is subsequently exchanged for an oxidized PQ molecule from a pool in the membrane. At the cytochrome b_6/f complex the reduced Q_BH_2 is oxidized again, and the electrons are transported via plastocyanin towards the lumen side of PS I. The transport across the membrane through the PS I reaction center requires light energy, analogous to the transport in PS II. The absorption maximum of the reaction center chlorophylls is different: 680 nm in PS II, and 700 nm in PS I. Via ferredoxin, the primary acceptor of PS I, $NADP^+$ is subsequently reduced to NADPH. This is the reducing agent used in the CO_2-fixation reactions.

1.3.3 The D1 protein of photosystem II - structure and function

The complete photosystem II, including the oxygen evolving complex, contains about 20 polypeptides. Their sizes range from 3 to 47 kD. Recently evidence was provided that the reaction center of PS II, the smallest complex capable of the primary charge separation upon illumination, consists of D1, D2 and cytochrome b_{559} (Nanba and Satoh 1987, Deisenhofer et al. 1984 and 1985, Michel and Deisenhofer 1988, Ghanotakis et al. 1989, Gounaris et al. 1989) and possibly another small protein (Ikeuchi and Inoue 1988). In Fig. 1.2 a representation of the photosynthetic reaction center proteins D1 and D2 is given.

The catalytic function of PS II is the electron transport from water to the secondary acceptor Q_B. After complete reduction Q_BH_2 is exchanged with an oxidized Q_B from the plastoquinone pool located in the thylakoid

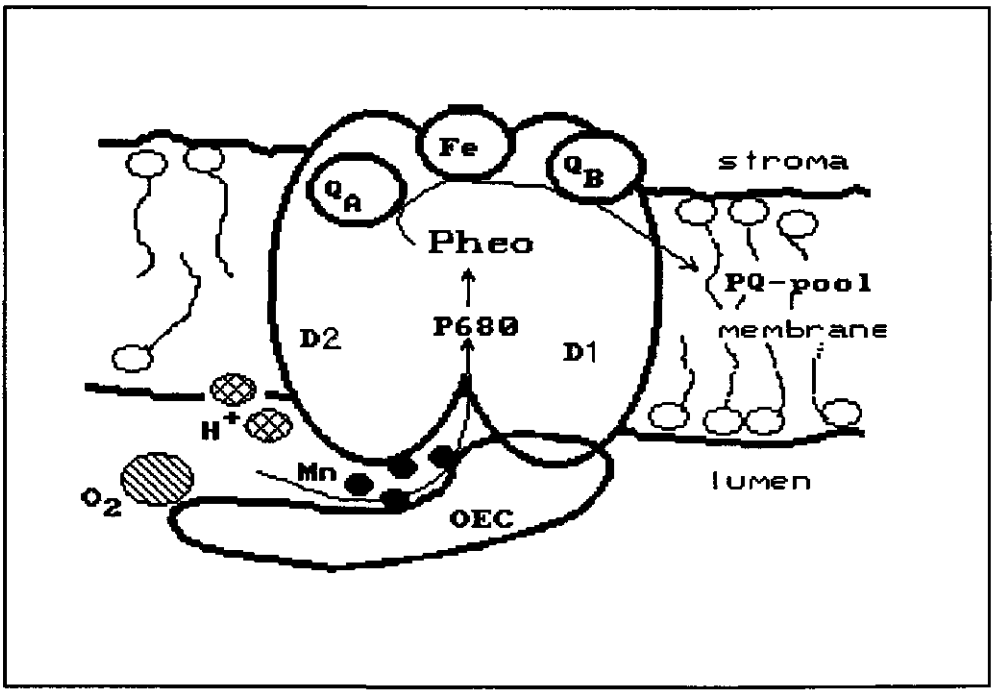


Fig. 1.2. The reaction center proteins D1 and D2. Binding sites for the acceptors and the non-heme iron are indicated. Arrows indicate electron transport from water via the OEC-Mn atoms, P_{680}, pheophytin, Q_A, possibly Fe, and Q_B to the PQ-pool.

membrane. The Q_B-binding protein (Ellis 1977) is one of the most studied plant proteins (review: Kyle (1985)). It is usually indicated as the D1 protein (Chua and Gillham 1977), as the herbicide binding protein, or according to its size as the 32 kD protein. The turnover rate of D1 is high (Ellis 1977, Mattoo et al. 1981). It is part of the PS II reaction center (Deisenhofer et al. 1985), regulates the electron transport at the PS II acceptor side (Mattoo et al. 1981), provides the Q_B-binding site, binds the so-called PS II herbicides (Pfister and Arntzen 1979), and is involved in

photoinhibition (Arntz and Trebst 1986). It is coded by the psbA gene, which is located on the chloroplast DNA (Zurawski et al. 1982).

The hydrophobicity plot of the D1 protein was initially thought to indicate that seven membrane spanning helices were formed (Rao et al. 1983), but later data were explained as forming only five (Trebst 1986). A scheme of the protein is drawn in Fig. 1.3.

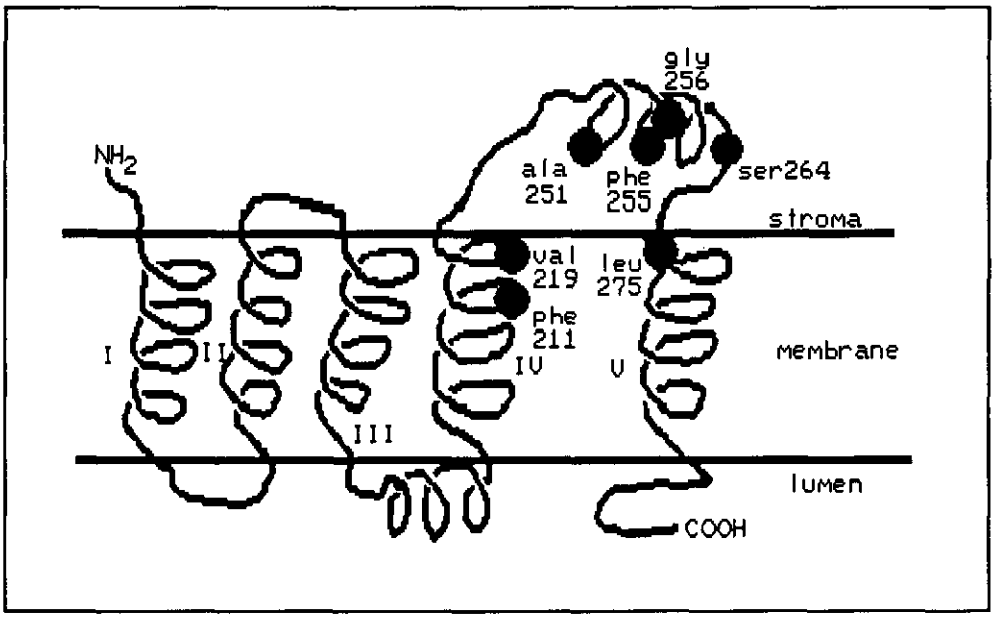


Fig. 1.3. The five membrane-spanning helices of the D1 protein. The filled balls indicate amino acid residues which are involved in triazine-resistance in plants (see Table 5.1).

The binding site for Q_B and herbicides is located between helices 4 and 5 (Trebst 1987). The amino acid residues forming the binding niche are known, but an exact steric model of the shape of the pocket is very hard to establish. There is evidence, though, that distinct amino acids are involved in inhibitor binding (Trebst 1987, Dostatni et al. 1988).

1.4 Modes of action of herbicides

Herbicides are compounds, mostly synthetic, which are used to kill or injure plants, in order to lower the level of competition that crop plants suffer from the presence of weeds. Since several decades a wide variety of chemicals has been used to control weed growth in crops. The first agent to be used in weed control, in 1932, was DNOC. It was soon followed by growth-hormone analogues of the phenoxyacetic acid type. These latter compounds are still in use as herbicides, amongst others because they are relatively harmless to insects and animals. In plants, they induce uncontrolled growth, leading to death of the plants. In the 1950's other types of herbicides became available: the urea-, triazine- and bipyridinium-compounds. These herbicides have in common the potency to inhibit a very plant-specific process: photosynthesis.

About 250 different weed controlling chemicals are now commercially available. Half of these compounds act directly on photosynthetic processes, and one third influences cell division and/or plant growth. The other 10 to 15 % act by deregulating or blocking various other metabolic processes like respiration or biosynthetic pathways as carotenoid-, lipid- or amino acid- biosynthesis.

The selective effect of herbicides, which increases their value for most applications, can be based on either physical or biochemical features, or a combination of both. Physical factors which may influence the effectiveness are the formulation, the technique, time or place of application, the degree of adsorption to the soil, or the structure of the plant. The biochemical basis for a selective effect can be any detoxification mechanism, like e.g. the natural resistance of maize to many triazine compounds. This resistance is caused by enzymatic conjugation of the triazine compound with glutathione, in combination with enzymatic breakdown of alkyl-substituents and non-enzymatic hydrolysis of the chloride-atom (Shimabukuro et al. 1970).

The modes of action of different photosynthetic herbicides is described in part in Paragraph 4.1. Many reviews are available on this subject (Moreland 1980, Fedtke 1982, Van Rensen 1988 and 1989).

1.5 Triazine resistance in weeds

Since 1970 many biotypes of common weeds have been found to be resistant to triazine-type herbicides. Especially in areas where the persistent compounds, like e.g. atrazine, had been used for a longer period, the occurrence of resistant plants became a practical problem (Ryan 1972, LeBaron and Gressel 1982).

Resistant plants and algae were shown to be different from the wildtypes not only in the resistance characteristics, but also in other properties. The lipid composition was found to be altered, as was the chlorophyll *a/b* ratio (Pillai and St. John 1981, Burke et al. 1982, Vaughn and Duke 1983, Chapman et al. 1985). The electron transport properties between the acceptors Q_A and Q_B were also affected in the resistant biotypes, which seemed an obvious explanation for the observation of a decreased growth performance (Bowes et al. 1980, Haworth and Steinback 1987, Erickson et al. 1989). Other authors recently proposed that a decrease in electron transport alone could not fully account for the decrease in the rate of photosynthesis in some herbicide-resistant biotypes (Jansen et al. 1986, Jursinic and Pearcy 1988, Ireland et al. 1988). Indeed, the *Chenopodium album* biotypes used in our experiments could not be distinguished on sight under the growth conditions used. Nevertheless, the difference in sensitivity to triazine compounds was about 1000-fold.

Closer examination of resistant biotypes of higher plants led to the discovery of a mutation in the D1 protein (Hirschberg and McIntosh 1983, for an overview see Chapter 5 of this thesis). The observed mutation from serine in the wildtype to glycine in the mutant was proved, at least in cyanobacteria, to be the only factor responsible for the increase in resistance, by transforming them with a genetically engineered psbA gene (Golden and Haselkorn 1985).

1.6 Outline of the study

Various important herbicidal compounds inhibit the photosynthetic electron transport at a site located on PS II. Although the mechanism of

action of these compounds is generally accepted to be a competitive interaction with the intrinsic PS II electron acceptor Q_B (see e.g. Pfister and Arntzen 1979, Trebst 1987), and many quantitative structure-activity analyses have been made (Hansch and Deutsch 1966, Trebst and Draber 1979, Huppatz and Phillips 1987a-c, Šoškić and Sabljić 1989), little is known about the interactions at the molecular level between herbicide and binding environment. This study was undertaken in order to obtain more information about the factors which influence the binding and release kinetics of some of these PS II herbicides.

A method is described to determine the exchange parameters of the PS II herbicides with the intrinsic electron acceptor Q_B at their common binding environment. This is achieved by measurements of flash-induced oxygen evolution. The amount of oxygen which is released upon illumination can be measured with a sensitive platinum electrode.

The herbicides used were DCMU, atrazine and ioxynil as examples of widely used PS II herbicides. Structural formulas are given in Fig. 1.4.

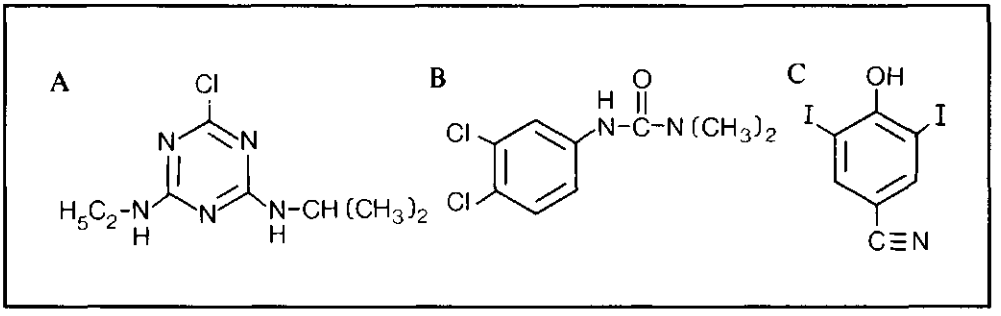


Fig. 1.4. Structures of various PS II electron transport inhibitors used in this study. A = atrazine, B = DCMU, C = ioxynil.

Also several non-commercially available cyanoacrylate compounds were used. These were kindly provided by Dr. J. Phillips. They were two pairs of stereo-isomers, showing large differences in inhibition properties within each pair. The structural formulas of the compounds are drawn in Fig. 1.2.

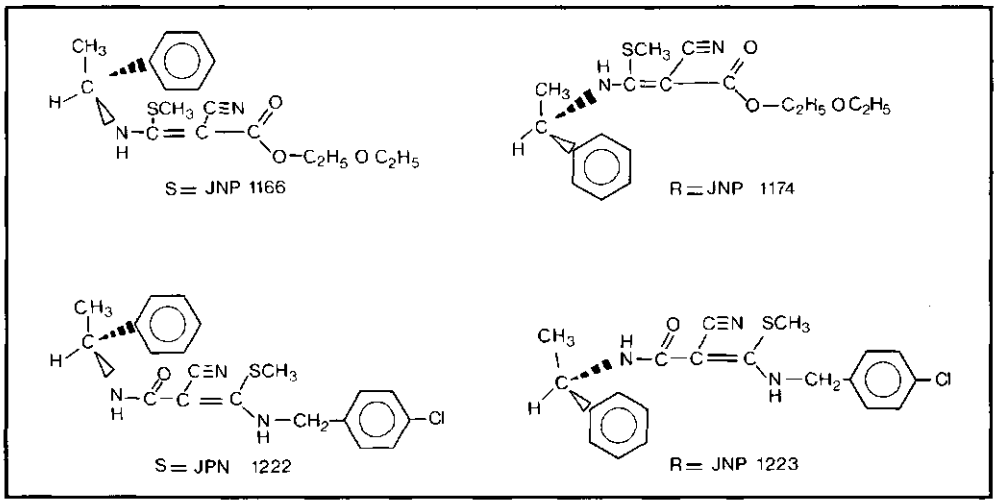


Fig. 1.2. Structure of the cyanoacrylate inhibitors. JNP1166 and JNP 1174 form one pair of isomers, JNP 1222 and JNP 1223 the other.

A biotype from the common weed lambsquarters (*Chenopodium album* L.) resistant to many triazine-class herbicides was used as a model system, in combination with the wildtype. The mutation causing the resistance in these plant species was determined on the level of nucleic acid by sequence analyses of the DNA and the mRNA.

2 Materials and methods

2.1 Introduction

In this Chapter the plant material is described, as well as the methods used to isolate chloroplasts or nucleic acids. For some standard procedures a reference will be given to methods published before. An outline is given of the measurements of flash-induced oxygen evolution and fluorescence induction. Furthermore short introductions are given on the molecular-biological procedures that have been used. For more details on the sequence analyses performed see Chapter 5.

2.2 Plant material

All plant material was grown in growth chambers, in order to obtain constant quality throughout all seasons. Pea (*Pisum sativum* L. cv. Finale) and lambsquarter (*Chenopodium album* L.) leaves were harvested from 4-6 weeks old plants. Seeds of the triazine-resistant biotype of *Chenopodium album* were collected in the eastern part of The Netherlands. Photosynthetic properties and growth performance of this biotype have been described before (Jansen et al. 1986).

The growth conditions were the same for all plant species. Temperature was kept constant at 19 °C day and night. The light period was 14 h per day, light intensity 45 W m^{-2} photosynthetically active radiation. Relative humidity was regulated at 65 %.

Unless stated otherwise the experiments were carried out with isolated, frozen thylakoids as a model system to measure oxygen evolution. The advantage as compared to using freshly isolated chloroplasts is a better reproducibility of the experiments, because multiple measurements could be carried out with a single batch of chloroplast material.

2.3 Chloroplast isolation

Broken chloroplasts were isolated from leaves of 4-6 week old plants. Usually 20 g (fresh weight) was used to obtain an amount of chloroplasts sufficient for 10 days of experiments. On a single day about 25 related measurements could be carried out.

Freshly harvested leaves were washed once with cold distilled water and ground in a Sorvall Omnimixer for 5 s in isolation and reaction medium (IRM). The homogenate was filtered through 2 layers of nylon cloth. Chloroplasts were collected by spinning for 60 s at 2500 g. The pellet was suspended in 50 ml shock medium (SM), to break the chloroplasts by means of an osmotic shock. The thylakoids were pelleted by centrifugation for 5 min at 1000 g, resuspended and diluted to a final concentration of 0.5 mg chlorophyll/ml in IRM. Total chlorophyll concentration was determined spectrophotometrically according to the method of Bruinsma (1963). Aliquots of 0.5 ml were stored at -80 °C, and slowly thawed on ice prior to use. The thylakoids were dark adapted for at least 1 h and used for measurements not later than 6 h after thawing.

2.4 Electrode used to measure flash-induced oxygen evolution patterns

The flash induced oxygen production was measured with a platinum-silver type of electrode (Joliot 1972). No absolute amounts of oxygen produced per chloroplast could be measured, only relative heights of peaks as a result of subsequent flashes are detected.

Our laboratory-designed equipment was constructed for measurements using a small amount of isolated chloroplasts, which could be deposited in a very thin (mono)layer directly on the platinum anode surface. Chloroplasts were allowed 5 to 10 minutes to settle on the platinum surface. For every measurement an amount of chloroplasts equivalent to 7.5 μg chlorophyll was used.

The setup assures a direct measurement, avoiding the necessity of diffusion of produced oxygen through a separating membrane. It obviously decreases the response time. A dense chloroplast suspension, as

used in several other types of oxygen measurement systems, may cause problems. For instance, in electrodes with a large thylakoid chamber the light intensity must be high in order to excite all thylakoid material, causing a high probability for double hits in the top layers and for misses at the bottom ones. The disadvantage of using a small amount of plant material is, of course, a weak signal, but this could be overcome using a sensitive low-noise amplifier, in combination with sufficient insulation from interfering electric and magnetic fields. In Fig. 2.1 a schematic representation of the electrode is given.

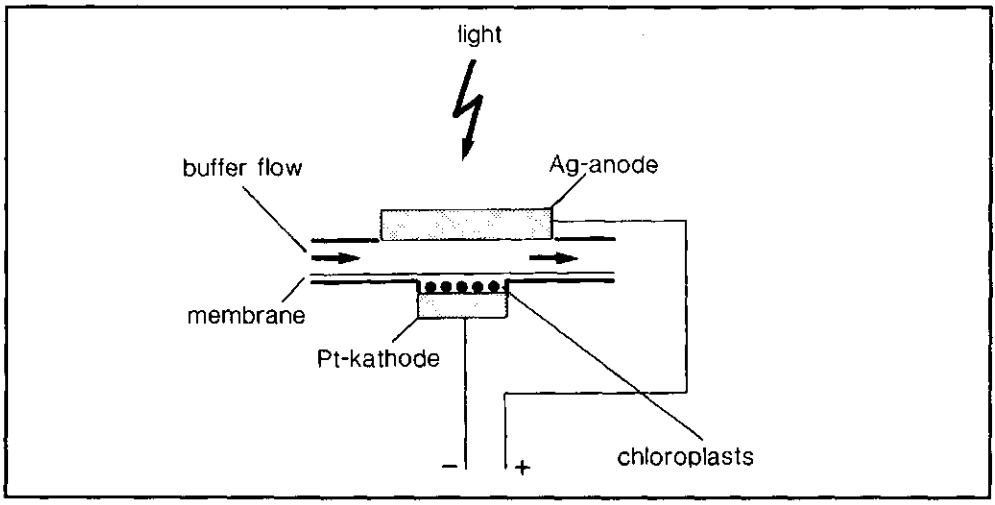


Fig. 2.1. Electrode for measurement of flash-induced oxygen evolution.

Flashes with a duration of about 8 μs were fired with a Xenon lamp. A Schott BG23 blue filter was used in order to minimize excessive red tailing effects. The flash train for measurement of oxygen production consisted of a series of 10 flashes spaced 0.1 to 4 s apart. For data recording and processing a Nicolet digital storage oscilloscope was used in combination with a HP-86 computer. Calculations and data fittings were carried out with the aid of a (IBM-XT or -AT compatible) personal computer.

2.5 Isolation and identification of plant DNA and RNA

For a sequence analysis a certain amount of nucleic acid is required. The amount is dependent on the method used. When the fragment to be analyzed is first cloned in a plasmid, the amount needed is significantly smaller than for the direct method described in Chapter 5.

2.5.1 Simultaneous isolation of DNA and RNA from fresh or lyophilized leaves

Isolation of nucleic acids can be achieved via a rapid procedure, yielding DNA and RNA at the same time (Murray and Thompson 1980, Taylor and Powell 1982). These can subsequently be separated by CsCl gradient centrifugation. The method is based on precipitation of nucleic acids in high salt solution in the presence of CTAB (cetyl trimethyl ammonium bromide), long hydrophilic alkyl molecules with a strongly polar head group, by inducing nucleic acid complex formation. The complex formed can easily be pelleted.

Since the *Chenopodium album* alteration causing triazine-resistance was to be determined, both the mutant and the wild type were analyzed. Of both biotypes, 10 g (fresh weight) of leaf tissue was harvested. The plant material was frozen in liquid nitrogen and ground to a fine powder in a pre-cooled mortar. After transfer to a glass tumbler, 0.2 ml β-mercapto-ethanol and 10 ml boiling extraction buffer (EB) were added. The mixture was transferred to a water bath at 55 °C and stirred gently until its temperature had increased to 50 °C. The solution was then transferred to a screw cap tube, an equal volume of chloroform/iso-amyl-alcohol 24/1 was added, and the contents were shaken gently (to avoid shearing) to mix the phases.

Centrifugation for 10 min at 4000 g at 20 °C was sufficient to separate the layers. The upper (water) phase was transferred to a clean tube, 10 % of the volume of precipitation buffer 1 (PB1) was added and extraction and centrifugation were repeated. An equal volume of precipitation buffer 2 (PB2) was added to the water phase, and after

mixing allowed to stand at room temperature for 15-30 min. Nucleic acids were pelleted by centrifugation for 10 min at 13000 g and 20 °C, resuspended in 20 ml 1 M CsCl-solution and carefully loaded on a cushion of 2 ml 5.7 M CsCl-solution in 12 ml centrifuge tubes. DNA and RNA were separated by spinning in a swing-out rotor for 12 h at 120,000 g at 20 °C.

As a consequence of the double helix structure, the floating density of DNA is slightly lower than that of RNA. The density of 5.7 M CsCl assures that DNA bands at or just below the interface, while RNA pellets through the cushion. The DNA-containing fraction (about 2 ml per tube) was diluted with 2 volumes of TE. The RNA pellet was drained and dissolved in 1 ml TE-buffer. The nucleic acids were then precipitated by adding 1/10 volume 3 M NaAc pH 5.2 and 2 volumes of ice-cold ethanol, and allowed to stand for 15 min at -80 °C. Centrifugation for 10 min at 15000 g pelleted the material, which was then taken up in 100-200 µl TE. Repetition of the precipitation, in combination with washing of the pellet with ice-cold 70 % ethanol, was applied to further purify the nucleic acids from polluting salts. If necessary, proteinaceous contaminations can be removed by phenol-chloroform extraction in combination with proteinase treatment. If necessary, the DNA can be treated with (DNAse-free) RNAse-A or -T1, to digest residual RNA.

The concentrations of DNA and RNA were determined spectrophotometrically. At 260 nm, a concentration of 50 µg/ml DNA or 40 µg/ml RNA (in a quartz cuvette with 1 cm optical pathlength) corresponds to 1 O.D.-unit. Using the described method, from 10 g of leaf material 100 to 150 µg DNA and 3 to 4 mg RNA could be isolated. This amount is sufficient for at least 10 separate DNA- and 50 RNA- sequencing reactions.

The DNA was sufficiently purified to be cut with restriction endonucleases by one more precipitation. A problem can be the presence of RNA-digesting enzymes in the preparation. As long as the nucleic acids are dissolved in the CsCl solution or in ethanol, RNAses are showing very little activity. After further purification care was taken to avoid enzymatic RNA breakdown by using sterilized pipette tips and reaction vessels.

2.5.2 DNA and RNA gel electrophoresis

Nucleic acid fragments can be separated according to their sizes by electrophoresis on different types of gels. Under the influence of an electrical field, the negatively charged material moves through the gel. The most common system is the use of agarose gels. The agarose, forming a network of linked and intertwined molecules, slows down the movement of the large nucleic acid molecules. The smaller the fragment, the higher the mobility in the gel.

The DNA bands can be visualized by staining the gel with ethidium bromide. This compound binds strongly to double helical nucleic acids, fluorescing strongly in the red region upon illumination with UV light. The ethidium bromide is often already included in the gel. The separation of DNA and RNA fragments was carried out according to standard procedures as described in a laboratory manual (Maniatis et al. 1982).

Gels used for separation of DNA fragments usually contain less agarose than RNA gels. The double strand material moves slower, and thus requires less agarose. Depending on expected fragment sizes, 0.6 to 2 % of agarose is used. RNA gels differ from DNA gels in that they contain formaldehyde and formamide. These are used to linearize the often entangled and self-annealed RNA-molecules and achieve correct separation according to their lengths.

2.5.3 Blotting and hybridization of DNA and RNA gels

Identification of fragments can be achieved in several ways. The easiest is to load a MW-marker containing fragments of known molecular sizes on the gel together with the sample. This method permits an estimation of the fragment sizes in the sample, but of course cannot discriminate between different pieces of DNA of about the same length. A method which is more laborious but gives consistent information is hybridization with a specific probe. The probe can be a small, synthetic single strand DNA fragment. It can recognize a complementary single strand DNA or RNA sequence and 'stick' to it. When the probe is

radioactively labeled, it can be spotted by autoradiography. The gel cannot be used directly for hybridization with a probe. First the nucleic acids must be transferred to nitrocellulose paper. After this blotting the actual hybridization can be performed. Single stranded nucleic acids are bound very tightly to the nitrocellulose by baking the blot for 2 h at 80 °C. The blotting method is based on transportation of the RNA or DNA by sucking a high-salt solution through the gel and the overlaid nitrocellulose paper. Double strand DNA must be denaturated before blotting, because blotting and hybridization can only be achieved successfully with single strand material. Denaturation can be performed by incubating the gel in a strongly alkaline solution, followed by neutralization. The blotting procedure is the same for DNA and RNA gels. After blotting, the gel can be stained with ethidium bromide in order to check if the nucleic acids have been removed quantitatively.

The procedure followed was according to Maniatis et al. (1982). DNA gels were denaturated in denaturation solution (DS) for 30 min and neutralized in neutralizing solution (NS) for another 30 min. Blotting time was 12 h, using 20 x SSC as the salt solution. After blotting the nitrocellulose paper was rinsed in 5 x SSC, air dried at room temperature for 15 min and vacuum dried at 80 °C for 2 h to bake nucleic acid to paper.

After drying the blot is ready for hybridization with any single strand probe. The nitrocellulose paper will bind any added DNA or RNA very strongly. To avoid non-specific hybridization of the probe the blot must be coated with nucleic acids, e.g. salmon sperm RNA or tRNA. This will cover all unoccupied binding sites of the paper. When the labeled probe is added, it can only bind to a complementary DNA or RNA sequence.

The prehybridization was carried out with 8 ml hybridization solution, which contains RNA and various salts. Care was taken to remove all air bubbles and seal the bag tightly. It was incubated in a gyratory shaker at room temperature for at least 3 h, then the solution was replaced and primer (about $3 * 10^6$ cpm) was added. The hybridization was continued for at least 4 h at room temperature with gentle shaking. After hybridization, the paper was washed several times in 4 x SSC and the level

of radio-activity was estimated. In some cases, when a high activity indicated presence of non-specifically bound probe, the paper was washed in 2 x SSC and/or at an elevated temperature (40 °C). Subsequently the paper was dried briefly and wrapped in plastic film, and a Röntgen film was exposed at -80 °C for 4 to 24 h.

2.5.4 A rapid method for DNA sequencing

A preparation of dsDNA, e.g. plasmid DNA, can be sequenced very rapidly using a commercially available sequence analysis kit. First the DNA is denaturated, then primer is annealed to the single strand template. Either a labeled primer or labeled dATP can be used, or even both to obtain a stronger signal. However, when instead of plasmid DNA total genomic DNA is sequenced, use of labeled dATP may cause appearance of unspecific signals as the result of unspecific hybridization.

About 20 µg total DNA was dissolved in 8 µl TE and denaturated with 2 µl 2 M NaOH for 10 min at room temperature. The ssDNA was diluted with 7 µl H_2O and precipitated with 3 µl 3 M NaAc pH 5.2 and 60 µl cold ethanol for 15 min at -80 °C. After centrifugation during 10 min at 15000 g the pellet was washed in 120 µl cold 70 % ethanol, spun down, vacuum dried and resuspended in 10 µl H_2O. Subsequently 5 µl (about $5*10^6$ cpm) primer and 2 µl annealing buffer (containing $MgCl_2$ and DTT) were added. The primer was hybridized by incubation for 20 min at 37 °C and 10 min at room temperature. Then 3 µl labeling mix (dGTP, dCTP, dTTP) and 1 µl dATP (^{32}P-labeled at the α-position) or the same amount of cold dATP (when a fluorescent or radioactive primer is used) were added. With 2 µl 1.5 u/µl T7-polymerase the DNA was incubate for 5 min at room temperature. In the next step the mixture was divided and 4.5 µl added to 2.5 µl of each of 4 dideoxy mixes containing a ddNTP. Incubation for 5 min at 37 °C was sufficient for strand synthesis, and the reaction was stopped with 5 µl stopmix (bromophenol blue, xylene cyanol). Before loading the samples on the 6 or 8 % PAA (polyacrylamide) gel, template and copy strand were separated by incubation of 3 µl of each sample 2 min at 80 °C, the rest of the sample was stored at -20 °C. The gel

was run for 2 h at 1250 V, and the same samples were reloaded when the bromophenol blue had reached the bottom of the gel and run for another 2 h. Finally, the gel was wrapped in plastic film and a Röntgen film posed.

i.5 A rapid method for RNA sequencing

A RNA preparation can be sequenced by annealing a primer and ıthesizing the complementary DNA strand with reverse transcriptase. ter separation of the strands the copy strand can be read on the ıuencing gel. The method is essentially the same as when a DNA nplate is used, except for the fact that, of course, reverse transcriptase ıst be used instead of T7-polymerase.

To 50 µg RNA in 5 µl TE 5 µl (about $5*10^6$ cpm) primer was added, l RNA and primer were coprecipitated and washed. Then 2.5 µl ıealing buffer (250 mM Tris-HCl pH 8.2, 0.3 M NaCl, 50 mM DTT) was ded, and annealing completed by floating the mixture in 200 ml water 68 °C, slowly cooling it down to 20 °C in 30 minutes.
e mix was put on ice with 4.5 µl R-buffer (250 mM Tris-HCl pH 8.2, 0.5 NaCl, 30 mM $MgCl_2$, 50 mM DTT). Then 2 µl dNTP-mix (2 mM of each 4 dNTP's in R-buffer) and 2 µl 15 u/µl AMV reverse transcriptase were ded. From this mix 4 µl was incubated with 1 µl of each of 4 dideoxy ×es (0.8 mM ddNTP) for 20 min at 45 °C. After addition of 1 µl TP-mix the vessels were incubated for 15 min at 45 °C, to complete the ction. The template was digested by incubating 15 min at 45 °C with 1 (DNAse-free) RNAse (A or T1).

The reactions were stopped with 21 µl stop-mix (bromophenol blue, ene cyanol) and boiled for 3 min before loading 2-12 µl on 6-8 % A-gel. The gel could be reloaded after 2 h of electrophoresis, after ich the films were exposed.

2.6 Chlorophyll *a* fluorescence induction measurements

Chlorophyll *a* fluorescence induction was measured as a control of the resistance of plants to triazine- and urea-type herbicides. Several freshly harvested leaves of triazine-resistant and -sensitive *Chenopodium album* plants were incubated in 50 ml distilled water, a solution of DCMU (1 μM) or atrazine (1, 10 or 100 μM). Fluorescence induction curves were measured with a Walz fluorometer, according to the method described before (Van Rensen and Spätjens 1987). The leaves were incubated in the dark for at least 1 h, submerged in the herbicide solutions. The time base for the fluorescence curves was 2 s. The leaves were exposed to the weak measuring beam at the time the actinic light was switched on. After 2 s a saturating white light pulse of 0.7 s duration was given, in order to determine the maximal fluorescence. This value was used to normalize the patterns, in order to facilitate qualitative comparison. Results of the measurements are given in Chapter 6.

2.7 Buffers and solutions used

IRM : 0.3 M sorbitol
50 mM Tricine-KOH pH 7.5
100 mM KCl
10 mM NaCl
2 mM $MgCl_2$

SM : 10 mM Tricine-KOH pH 7.6
10 mM NaCl
5 mM $MgCl_2$

DS : 0.5 M NaOH
1.5 NaCl

NS : 1 M Tris-HCl pH 8.0

1.5 M NaCl

EB : 0.1 M Tris-HCl pH 8.0
1.4 M NaCl
2 % (w/v) CTAB
20 mM EDTA

PB1 : 10 % (w/v) CTAB
1.4 M NaCl

PB2 : 50 mM Tris-HCl pH 8.0
1 % (w/v) CTAB
1 % (v/v) β-mercapto-ethanol
10 mM EDTA

1 M CsCl: 50 mM Tris-HCl pH 8.0
50 mM NaCl
1 M CsCl
5 mM EDTA

5.7 M CsCl: 50 mM Tris-HCl pH 8.0
50 mM NaCl
5.7 M CsCl
5 mM EDTA

TE : 10 mM Tris-HCl pH 8.0
1 mM EDTA

20 x SSC : 3 M $NaCl_2$
0.3 M Na-Acetate pH 7.0

3 Determination of parameters used to characterize flash-induced oxygen evolution in isolated thylakoids

3.1 Introduction

In plant photosynthesis light quanta are used to induce transmembrane electron transport from H_2O to plastoquinone through PS II. Water molecules are the natural electron donors, the final acceptor is $NADP^+$. The electron transport induces indirectly the formation of an electrochemical potential gradient of protons across the thylakoid membrane. Under our experimental conditions this potential gradient is mainly composed of a pH gradient, which is the driving force for ATP-synthesis. The reduced NADPH is subsequently used in the Calvin cycle, where eventually carbon dioxide is fixed to form organic sugar compounds.

3.1.1 Electron transport reactions around photosystem II

The reaction sequence for water oxidation is described by the model of Kok et al. (1970). According to this model, an excited PS II - RC (reaction center) is able to extract electrons from the OEC (oxygen evolving complex) in a 4-step sequence. The intermediary so-called S-states are formed by repeated extraction of an electron from a specialized environment of the water splitting system. The most reduced state is indicated as S_0, the others respectively as S_1 to S_3. The water splitting complex, containing 3 extrinsic polypeptides with approximate molecular weights of 18, 23 and 33 kDa, respectively, is located at the inner side of the thylakoid membrane (for a review see Renger (1987)). This is the site where water is split and molecular oxygen is released. The evolution of one molecule of O_2 requires cooperation of 4 oxidizing equivalents. The

complex contains 4 Mn atoms, probably held in position by the large 33 kDa subunit. They can successively be oxidized by electron extraction by PS II when short saturating flashes are fired. The Mn atoms are proposed to be oxidized from the Mn^{3+}- to the Mn^{4+}-state (Dekker et al. 1984a), although the possibility that Mn^{2+}- Mn^{3+}-transitions are involved cannot be excluded (Vincent and Christou 1986). Also a model involving both Mn^{2+}-Mn^{3+} and Mn^{3+}-Mn^{4+} transitions has been proposed (Saygin and Witt 1987).

The electron donor to the PS II - RC is Z. This donor has been identified as a tyrosine residue of the D2 protein which, together with at least D1 and cyt b_{559}, forms the RC. When the RC is excited an electron is transported from Z to the acceptor side of PS II, which is populated with a primary (Q_A) and a secondary (Q_B) quinone. The donor system then oxidizes the OEC, and thus gets rereduced.

At the acceptor side the primary one-electron acceptor Q_A is reduced on each flash, transferring the electron to the secondary acceptor Q_B on a 100-200 μs timescale (Robinson and Crofts 1983). Q_B can be reduced twice and become protonated to form Q_BH_2, thus providing a 2-step gating mechanism (for reviews see Wraight (1983) and Crofts and Wraight (1983)). The reduced Q_BH_2 is subsequently exchanged within 10 ms for a Q_B from the quinone pool. Between both acceptors Q_A and Q_B an intermediate redox component, termed Q_{400}, is situated (Ikegami and Katoh 1973). This component was shown to be a high-spin Fe atom (Petrouleas and Diner 1986). The iron is proposed to be ligated to 4 histidine residues of the D1 and D2 proteins of the RC (Trebst 1987, Evans et al. 1988). It may be involved in electron transport between the quinones, at least in photosynthetic bacteria (Okamura et al. 1975). For a recent review on the function of Q_{400} see Diner and Petrouleas (1988). Recently it was suggested that Q_{400} is present in only about 50 % of the reaction centers (Jursinic and Dennenberg 1988), but at the same time a new quinone acceptor was introduced. In PS II particles the high-spin Fe-atom has been shown to oscillate between the Fe^{2+}- and Fe^{3+}-states, depending on the redox state of the artificial quinone acceptors and an as yet unknown protonizable group (Renger et al. 1987). The Fe^{2+}-form is proposed (Van Rensen et al. 1988) to be able to donate an electron to the semireduced quinone Q_B,

thereby inducing the formation of Q_BH_2 after a single flash in dark-adapted chloroplasts. Such a mechanism has been described as reductant-induced oxidation (Zimmermann and Rutherford 1986), and was observed when Q_B had been replaced by an artificial quinone. Although as yet not observed, the occurrence of such mechanism in PS II electron transport *in vivo* cannot be ruled out.

3.1.2 Oscillations in the flash-induced oxygen evolution

When oxygen evolution induced by short saturating flashes is measured, an oscillation pattern with period 4 is observed (Joliot 1968, 1972). This pattern can be explained with the Kok model by assuming miss and double hit parameters, symbolized by α and β, respectively (Kok et al. 1970). A certain fraction of the RC's proves to be unable to make a turnover, and thus gets out of phase with the bulk of the centers. Another 3 % fraction makes a double turnover, even when the flash duration is as short as 5 ns (Jursinic 1981). Even at saturating flash intensities, in chloroplast suspensions a certain amount of RC's is unable to make a turnover. This fact suggests that, apart from missing the antennae pigments, another mechanism must be held responsible for part of the misses that occur. A possible explanation is the localization of an electron on the RC, which decreases the possibility of the center trapping a second light quantum (Vermaas 1984).

The α-parameter has been assumed by most authors to be independent of the flash number. It represents a fraction of the RC's not making a turnover on certain flashes (Forbush et al. 1971, Joliot et al. 1971). Another model was based on the assumption that the value of α is not the same for all flashes (Delrieu 1974, 1983). Only a certain fraction of the centers coupled to an OEC in the S_2-state was held responsible for the occurrence of misses. This so-called α_2 was calculated to have a value of 0.5-0.7, whereas the α on other flashes was found to be 0. The specific α_2 was ascribed to an incapability to light-saturate the S_2 to S_3 transition. Bouges-Bocquet (1980) suggested that misses are due to back-reactions of

the S-states before a charge-stabilization can occur and to a loss of a fraction of the positive charge on the Mn-atoms during each 4-step cycle.

3.1.3 Modifications of the classical model

In this chapter a refinement of the Kok model is proposed, assuming a correlation between the miss parameters α_n ($0 \leq n \leq 3$), associated with the different S-states of the OEC, and the redox state of the quinone acceptor complex. After a dark period longer than about 10-20 ms, which is longer than the time necessary for the exchange of $Q_B H_2$ with Q_B (Stiehl and Witt 1969), the complex can exist only in the oxidized or the semireduced form. The probability for any RC to be closed and thus being incapable of trapping a light quantum, is higher when the acceptor side contains an electron as compared with the situation that the quinone complex is fully oxidized. This results in an adaptation of the S-state model, predicting alternately a relatively high and a low value for α on successive flashes.

The probability for a miss occurring on the S_0 to S_1 transition is denoted as α_0, the others as α_1, α_2 and α_3, respectively. It has been proposed that the presence of an electron located at the primary acceptor Q_A strongly decreases the probability of a stable charge separation upon light excitation. Furthermore, the α parameters may also be influenced by the kinetics of the S-state transitions. The reaction constants are not the same for all transitions (Dekker et al. 1984b, Renger and Weiss 1986, Renger 1988). The rate limiting step in water oxidation seems to be the release of oxygen from the niche provided by the 33 kD extrinsic protein (Plijter 1988).

The double hit parameter β is, at least partially, attributed to a dual turnover of the RC caused by a second light quantum during the tail of the actinic flash (Kok et al. 1970, Jursinic 1981). Also the heterogeneity of the PS II antennae population may be responsible for a percentage of the double hits, independent of the light intensity.

Another factor which influences the oxygen evolution pattern is the occurrence of an electron donor D, which is able to reduce the S_2-or S_3-state of the OEC (Vermaas et al. 1984). Based on EPR-measurements it

was assumed that this donor would be a quinone-like molecule (O'Malley and Babcock 1984, Diner and De Vitry 1984). Recently however, Barry and Babcock (1987) explained their EPR- and mass spectrometry data by suggesting both electron donors D and Z to be tyrosine residues of respectively the D1 and the D2 protein. This suggestion was verified by elegant site-directed mutagenesis experiments (Debus et al. 1988, Vermaas et al. 1988).

Two mechanisms are responsible for a time-dependent decay in the dark of the states S_2 and S_3 of the OEC to the stable S_1-state (Velthuys and Visser 1975). The first occurs when chloroplasts (or plants) are kept in complete darkness. This dark adaptation is a combination of several rather slow reactions, with half-times in the order of minutes. These processes result in the stabilization of the OEC in the S_1-state, which contains one oxidizing equivalent. The S_2-and S_3-states are slowly reduced, while S_0, if present, is getting oxidized. The electrons which are involved in these reactions may be exchanged with the proteinaceous environment. A second mechanism which has been proposed is the reduction of the S_2- and S_3-states by an electron donor D. This reaction is much faster than the dark-adaptation, displaying a half-time in the range of seconds. The donor molecules D are rereduced very slowly in the dark, with a half-time of at least several minutes. A single donor molecule can thus be considered to reduce an S-state only once during the flash train period used for the measurements. In fact, both mechanisms described are inducing a back-reaction of states S_3 to S_2 or S_2 to S_1. In this chapter, the term back reaction is reserved for the reduction of the S-states by donor D. The timescale for the dark-adaptation is so slow compared to this reaction, that its influence is negligible during the measuring periods.

In our model the donor D is the only time-dependent factor influencing the damping of the pattern. When the flash frequency is increased a higher maximum in oxygen evolution on flash 3 is observed, even when the other experimental conditions are exactly the same. This means a reaction occurring in the dark period between the flashes must be held responsible for this observation. Donor D seems able to account for this feature, since it is able to induce a back reaction of the S-states. Thus a higher oxygen response on flash 3, observed when the time between the

flashes is shortened, is due to a lower probability for a back reaction to occur between two consecutive flashes.

In summary, our modifications of the Kok-model are the assumption of 4 different miss parameters, and involvement of the donor D on the oxygen evolution patterns.

3.2 Results

The miss- and double hit parameters were calculated from the oxygen yields in a series of 10 saturating flashes. The data were normalized to a steady state production of 1 (a.u.) per flash. The flash frequency was varied from 0.25 to 10 Hz. A description of the electrode equipment is given in Paragraph 2.3.

3.2.1 Determination of the fraction D and its reaction constants

The reaction constant for reduction of states S_2 and S_3 by donor D, and the fraction of RC's in which it can occur, were determined from experiments in which the chloroplasts were preilluminated with 2 flashes. These preflashes are either fired 0.2 s after each other, followed by a time interval in which the back reaction can occur, or the time interval is spaced between both preflashes, after which the flash train is fired immediately. In the first case the time interval starts when most centers are in state S_3. In the other situation the back reaction of state S_2 can be studied. The oxygen production on the next flash, the first of a train of 10, is in both cases related directly to the amount of centers in state S_3. It can be used to calculate the amount of centers in which a back-reaction has occurred. In these centers the oxygen evolution is retarded one step, requiring another turnover. The time interval (Δt) between preflashes and flash train was varied.

In Fig. 3.1 A-C examples of oxygen evolution patterns are given. In case A almost no back reaction occurs, because preflashes and flash-train are spaced only 0.2 s apart. The fraction of centers in which a back

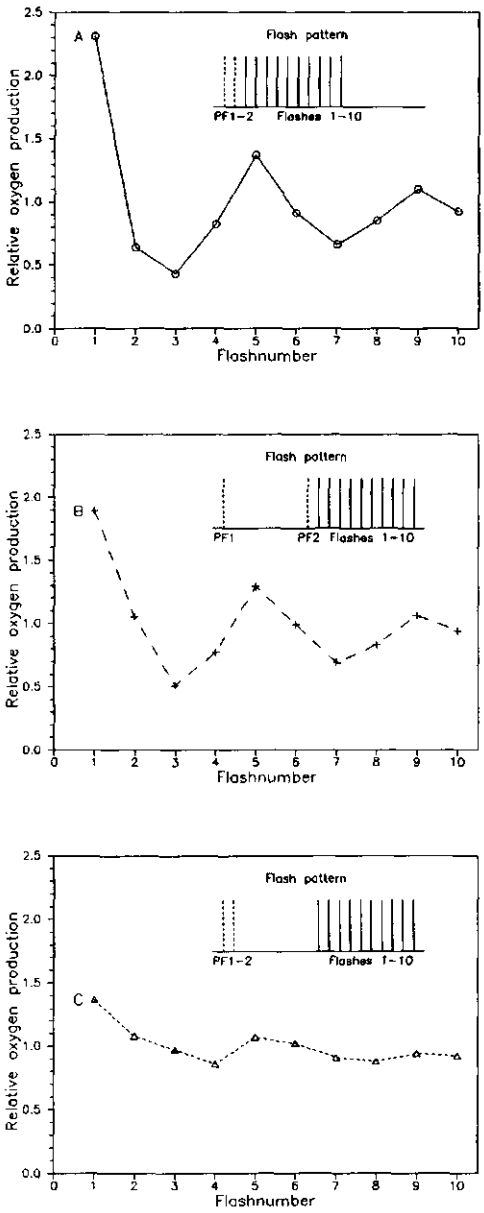


Fig. 3.1. Oxygen evolution patterns in pea thylakoids after illumination with 2 preflashes. A (O): Δt=0.2 s; B (+): Δt=10 s, S_2 to S_1 transition; C (Δ): Δt=10 s, S_3 to S_2 transition. The time interval Δt was varied between 0.2 and 10 s, flash frequency in the flash train 1 Hz. PF=preflash(es), Flashes 1-10=flash train.

reaction has occurred is indicated by the relative difference in peak height on flash 1 between traces A and B (S_2-decay) or A and C (S_3-decay).

Oxygen production on the first flash of the flash train decreases, when the dark interval between preflashes and flash train is extended. The decrease in height of this oxygen peak quantifies the amount of centers in which a back reaction has occurred. When state S_2 or S_3 of the OEC is reduced once to form S_1 respectively S_2, oxygen evolution is delayed to the 2nd instead of the 1st flash of the flash train, and thus the relative height of the first peak decreases.

In Fig. 3.2 the decrease in oxygen evolution after the first flash, which represents the back reactions, is expressed as a function of the time between the preflashes and the flash train. The 10 measuring flashes were given at a 1 Hz frequency, so at the end of the flash train all molecules D are supposed to have been oxidized by reducing either S_2 or S_3 on any flash. In this way, the net oxygen production is not influenced by the length of the time interval between preflashes and flash train.

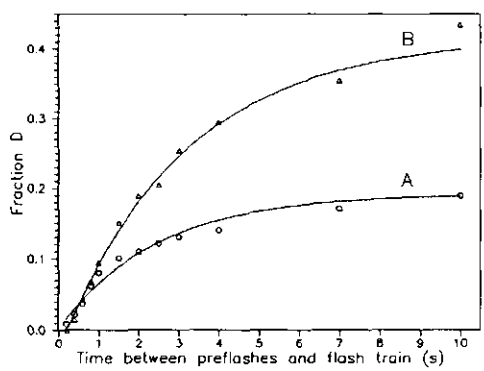


Fig. 3.2. Exponential curve fitting of estimated fraction D in pea chloroplasts. Fraction D is shown as a function of the time interval Δt between preflashes and flash train. In curve A the time interval was spaced between both preflashes, whereas in curve B both preflashes were given before the time interval Δt. This means that in curve A the S_2 to S_1 back reaction was measured, and in curve B the S_3 to S_2 decay. The curves are drawn as the best fit through averaged values of 5 experiments. Measurements started on t=0.2 s.

For the reaction of donor D with the S-states a simple exponential decay mechanism is proposed, resulting in the following equation :

$$[D_{ox}]_t = [D_{red}]^*(1-e^{-k_D^*t}) \qquad (3.1)$$

where $[D_{ox}]_t$ is the fraction of the RC's coupled to an oxidized donor molecule at time t, $[D_{red}]$ is the fraction of the centers containing reduced D after dark adaptation and k_0 is the reaction constant for the electron donation. Using Eqn. 3.1, parameters were calculated from the average values of at least 5 experiments with 12 different time intervals between preflashes and flash train. The results, obtained with chloroplasts isolated from pea, the wildtype and the triazine-resistant mutant of *Chenopodium*, are presented in Table 3.1.

A triazine-resistant mutant of *Chenopodium album* shows an alteration in electron transport properties as compared to the wildtype (Holt et al. 1983). This is caused by a change in the D1 protein, which is a part of the PS II - RC. The slight modification of this protein (see also Chapter 5) influences the equilibrium between reduced primary acceptor Q_A and semi-reduced Q_B, slowing down electron transport to the quinone pool (Pfister and Arntzen 1979). This feature makes these *Chenopodium* plants a suitable system to compare the influences of different electron transport properties on the various parameters of the model presented here, using pea chloroplasts as a well-characterized reference.

In Table 3.1 it is shown that both types of *Chenopodium* chloroplasts show only slight differences in apparent fraction D and reaction constants measured for the two back reactions. An average value of 30 % of the RC's is taken to be connected to a reduced donor after dark adaptation. An average value of 0.3 can be concluded for the reaction constant k_0. This value will be used in the calculations of α_n and β. In these calculations the back reaction is not separated into S_2 to S_1 and S_3 to S_2 decay.

Table 3.1. Determination of the fraction D and reaction constants for the reduction of states S_2 and S_3.
D_{red} = fraction of the RC's containing reduced donor D after dark adaptation,
k_D = reaction constant for the back reaction of D with the OEC,
$t_{1/2}$ = half time for the back reaction.

		D_{red}	k_D (s^{-1})	$t_{1/2}$ (s)
Pea	[a]	0.19	0.41	1.7
	[b]	0.45	0.32	2.2
Chenopodium S	[a]	0.26	0.28	2.5
	[b]	0.32	0.34	2.1
Chenopodium R	[a]	0.26	0.27	2.5
	[b]	0.34	0.33	2.1

[a] : for S_2 to S_1 reduction;
[b] : for S_3 to S_2 reduction.

In pea chloroplasts, a significant percentage of RC's seems to be connected to a donor that reduces the S_3-state rather than the S_2-state. If all donor molecules that can reduce S_2 are assumed to be able to reduce S_3 as well, still in some 25 % of the centers S_3 can be reduced by a donor not acting on S_2. In *Chenopodium* this was also observed, but to a much lesser extent. The reaction constants for S_3 reduction are comparable for the different plants used.

3.2.2 Miss- and double hit-parameters

As the miss parameter for centers associated with an OEC in the S_2-state is found to be zero (see below), the centers in this state after the first preflash are advanced to S_3 on the second. This reduces the error that is made by assuming all centers to be in either S_2 or S_3 after 1 or 2 preflashes, respectively. It appears to be very difficult, however, to get the RC's exclusively in either of these states using flash-induced turnovers. This means that always a certain amount of S_2 is present when the reduction of S_3 by D is measured, and vice versa. The values for α_n and β calculated below implicate that after 1 preflash about 10 % of the number of centers assumed to be in state S_2 is in state S_3 as a consequence of making a double turnover. After 2 preflashes the situation is found to be reversed, as the result of a combination of a miss and a single hit. However, these small percentages do not have much influence on the parameters calculated (fraction D and k_D), because these are in the same order of magnitude for states S_2 and S_3.

The fraction of RC's associated with an OEC in state S_0 after thorough dark adaptation, the miss parameters α_n and the double hit parameter β were calculated by fitting experimental and theoretical oxygen evolution patterns using a least-squares fitting method (Table 3.2). Donor D was assumed to cause an exponential decay of state S_2 to S_1 or S_3 to S_2 (see Eqn. 3.1). This decay was calculated as a function of the flash frequency, or the dark interval between 2 succeeding flashes. Initially, all parameters were free running. When it turned out that in general, comparing many independent experiments, S_0, α_0 and α_2 tended to approach 0, these parameters were fixed to be 0.

Table 3.2. Parameters used to describe flash-induced oxygen evolution in different plant species.
Parameters were calculated from mean values of at least 7 series of experiments with flash frequencies 0.25, 0.5, 1, 2, 4 and 10 Hz. Sum of quadratical differences between experimental and theoretical data (SD = $\Sigma_{1-10}(Y_{EXP}-Y_{TH})^2$) varied from 1 to 2.5 %.
S_0 = fraction of centers in the S_0-state after dark adaptation
β = double hit parameter
α_n = miss parameter for S_n to S_{n+1} transition with n=0,1,2,3. ($S_4 = S_0$).

	Pea	Chen.-S	Chen.-R
S_0	0	0	0
β	0.05	0.06	0.09
α_0	0	0	0
α_1	0.19	0.17	0.21
α_2	0	0	0
α_3	0.35	0.37	0.50

The S_0-fraction after thorough dark-adaptation was calculated to be 0 in all experiments. This means that in the dark all oxygen evolving centers are in state S_1, since S_2 and S_3 are reduced in the dark. We suggest that, under our conditions, this state is coupled to an acceptor complex containing a single electron on either Q_A or Q_B (see Paragraph 3.3). This situation can be represented as an equilibrium between $Q_A^-.Q_B$ and $Q_A.Q_B^-$. In the dark the complex will be predominantly in the situation $Q_A.Q_B^-$, which is the more stable.

A RC in this state is proposed to have a comparatively higher probability for not making a turnover upon illumination, resulting in a high value for α on the first flash. The best fits for α_n were obtained by choosing α_0 and α_2 to be 0, while the sum of α_1 and α_3 was about 0.5. The value for β is calculated to be 0.06 for pea chloroplasts, and a little higher for both *Chenopodium* biotypes.

In the dark, the RC's can be forced to an at least partly oxidized situation by incubation with ferricyanide (Ikegami and Katoh 1973, Wraight 1985, Renger et al. 1987). This incubation condition causes a shift in the values found for α_n. In Fig. 3.3 the results of incubation in oxidizing conditions are presented.

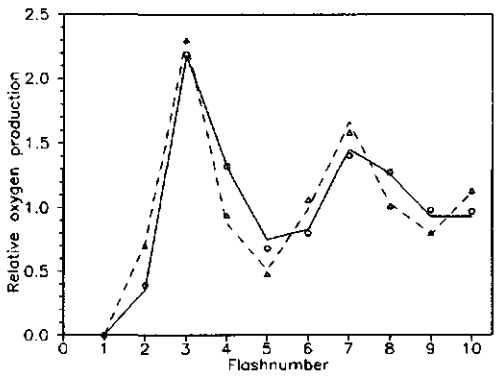


Fig. 3.3. Experimental and theoretical oxygen evolution patterns in pea thylakoids. Measurements in thylakoids treated with 2 mM FeCy (- - -) are compared to control measurements (———). Best fitting patterns are indicated with (Δ) and (O) for FeCy incubation and control, respectively.

The result of the incubation was a significantly altered oxygen evolution pattern (Fig. 3.3). When 2 mM FeCy was present no good fit could be obtained with high values for α_1 and α_3. When the misses were connected to the S_0- and S_2-states ($\alpha_1 = \alpha_3 = 0$, α_0 and $\alpha_2 > 0$), the fit was as good as in non-treated chloroplasts (Table 3.3).

It can be concluded from Table 3.3 that after the addition of FeCy a better fit (lower SD) is obtained when the initial setting is $\alpha_1 = \alpha_3 = 0$, instead of $\alpha_0 = \alpha_2 = 0$. As outlined above, this latter setting gives the best fit for the control. The fit of the FeCy-experiments in column 2 is as well

Table 3.3. Influence of FeCy-incubation on values of best fitting parameters.
FeCy = pea chloroplasts incubated in 2 mM ferricyanide for at least 30 min. SD = sum of quadratical differences = $\Sigma_{1-10}(Y_{EXP}-Y_{TH})^2$. The parameters which equal 0 in the table are set to 0 in the calculations. Flash frequency 1 Hz in all experiments. Column 1 and 3: $\alpha_0=\alpha_2=0$; column 2 and 4: $\alpha_1=\alpha_3=0$.

	FeCy		Control	
S_0	0	0	0	0
β	0.10	0.10	0.06	0.04
α_0	0	0.02	0	0.02
α_1	0.22	0	0.14	0
α_2	0	0.26	0	0.50
α_3	0.07	0	0.34	0
SD	0.023	0.018	0.017	0.046

as that of non-treated chloroplasts in column 3, i.e. when the non-zero values for the miss parameters are shifted to α_0 and α_2. For the control experiment, also the result of a fit using $\alpha_1 = \alpha_3 = 0$ is given. The fit is, as expected, poor. Moreover, the values found, especially the high α_2, seem non-physiological. The rather low value for α_0 after FeCy-incubation may be caused by a very low miss probability on the first flash. Also the sum of the misses is lower. Both findings can be the result of the artificial oxidation by incubation with FeCy. Also the slightly higher value of β can be caused by an electron deficit on the acceptor side, facilitating a double turnover on the first flash after dark adaptation.

3.3 Discussion

Especially the experiments in which ferricyanide is used to oxidize the acceptor side of the reaction center complex, show that the redox state of the acceptor complex has a distinct influence on the oxygen evolution patterns. From model calculations, fitting theoretical with experimental patterns, it appears that the Kok model has to be modified. Therefore some modifications are proposed: introduction of 4 miss parameters α instead of one, and the reduction by donor D of the states S_2 and S_3 of the OEC. The values found for the different parameters are evaluated and related to the proposed model.

Values for the half-time for the reduction of S_2 and S_3 by the auxiliary donor D have been reported in several papers. Babcock and Sauer (1973) calculated a half-time of 1 s, while Vermaas (1984) found 1.4 s. In our experiments the back reactions proved to be a little slower, with half-times ranging from 1.7 to 2.6 s (Table 3.1). The decay rates of the S_3-state were equal for all biotypes used. This indicates that the activity of the donor D is not influenced by the shift in equilibrium between $Q_A^-.Q_B$ and $Q_A.Q_B^-$, which in triazine-resistant mutants is supposed to be shifted to the Q_A^--side (Vermaas 1984). Especially in pea chloroplasts the reduction of the S_2 state is somewhat faster. This might indicate a slightly different mechanism for this reaction, which could also account for the apparent increase in total amount of reduced donor molecules available after dark adaptation. An explanation for these observations could also be a charge recombination between the reduced acceptor complex and the S_3-state. The measuring method does not allow discrimination between this charge recombination and a back reaction of S_3 with donor D. The result is a general value for all decay reactions of the S-states. The fraction of the RC's coupled to a reduced donor D after dark adaptation is about 30 %, which is in agreement with data from other authors (Vermaas et al. 1984).

The amount of RC's in the S_0-state after dark adaptation is found to approach 0. Other authors have assumed a fraction S_0 after dark adaptation of 20-30 % (Forbush et al. 1971, Joliot et al. 1971). This is an over-estimation, caused by the fact that no back reaction with donor D

was assumed to occur. This back-reaction can easily be mistaken for a certain fraction of state S_0, especially when the time between the flashes is longer than 1 s (Velthuys and Visser 1975). In that case in a certain fraction of the centers a back-reaction occurs. The OEC is reduced once there, and it seems as if the centers after dark adaptation started in a situation one step back compared to where they really were, in S_1.

The sum of the four α_n-parameters appears to be about 0.5 (Table 3.2). This means an average of 0.12 per S-state transition, and corresponds to the data of Vermaas (1984) and Delrieu and Rosengard (1987). The α on even flashes is low, that on odd flashes high. This is accounted for by assuming α to be dependent of the redox state of the quinone complex. As pointed out (Table 3.2) this introduces a 2-step oscillation. On account of the number of flashes fired, a semi-reduced quinone complex, and a high miss parameter, would be expected after an odd number of flashes. However, a high α is found after an even flash number: α_1 and α_3 are high. An explanation for this fact could be the existence of a semi-reduced complex $Q_A.Q_B^-$ even after long dark adaptation. The incubation on the electrode surface, needed for the chloroplasts to settle down, may be responsible for this reduction. Since the electrode reduces O_2 from the medium to OH^-, and measurements are carried out at pH 7.6, the experiments are done under anaerobic conditions. This may induce a reduction of the quinone acceptor complex. Also in intact leaves a dark reduction of Q_A by reverse electron flow could be observed under certain conditions, with luminescence and fluorescence techniques (Sundblad et al. 1988). Presence of a fraction of 25 % reduced Q_B even after prolonged dark adaptation was reported before (Wollman 1978, Rutherford et al. 1982, Demeter and Vass 1984, Vass et al. 1987). These observations suggest the possibility of the existence of a partially reduced acceptor complex under our measuring conditions. The equilibrium between $Q_A^-.Q_B$ and $Q_A.Q_B^-$ under these conditions determines the actual value for the miss parameters.

The value of α_1 may be slightly under-estimated, because relatively more reduced Q_B exists after dark adaptation. This means that on the first flash after dark adaptation a lower miss parameter could be expected. The

value for α_1, however, is calculated as the average of the miss probabilities for the flashes 1, 5 and 9.

By incubation with ferricyanide an electron can be extracted from the quinone-iron acceptor complex, to form $Q_A.Fe^{2+}.Q_B$ or even $Q_A.Fe^{3+}.Q_B$ already in the dark (Renger et al. 1987). Thus, at least one extra turnover is needed to get the situation $Q_A.Fe^{2+}.Q_B^-$, which in the untreated chloroplasts is supposed to exist in the dark. After dark incubation with FeCy, the best fit was shifted to lower values for α_1 and α_3, while α_0 and α_2 were increased. It was expected that α_1 and α_3 would be close to 0, because incubation with ferricyanide oxidizes the quinone-iron complex. However, a fraction of the centers may not get oxidized, as a result of the anaerobic incubation in a medium with pH 7.6 at the electrode surface during the last 5 minutes before the measurements. A mixed population of centers in states $Q_A.Fe^{3+}.Q_B$, $Q_A.Fe^{2+}.Q_B$ or $Q_A.Fe^{2+}.Q_B^-$ may exist, which makes unambiguous fitting very difficult. The differences in oxygen evolution patterns in these experiments proved though, that the acceptor side has a distinct influence on the occurrence of misses.

The double hit parameter β is not expected to be dependent on the flash number. The 2-electron gating mechanism in Q_B-reduction assures that upon a double turnover the quinone complex must cycle through both a semi-reduced and fully reduced state which is readily oxidized, starting in either of these. In either the first or the second stage of the double advancement an electron is present on the quinone complex, resulting in a higher miss parameter for one of both specific turnovers. So, if there is any dependency of β on the redox state at the acceptor side at all, it cannot be observed in our experimental set-up. In the model described here, the net effect is assumed to be an approximately constant value for β during the flash train.

In the triazine-resistant *Chenopodium*, the sum of the miss parameters is somewhat higher than in pea. This might be due to the shifted redox equilibrium of the quinone complex, which results in a higher fraction $Q_A^-.Q_B$ compared to $Q_A.Q_B^-$ (Vermaas 1984). This may cause more RC's to be temporarily closed, thus increasing α_1 and α_3. The β parameter is found to be slightly higher in the resistant biotype. We cannot explain this observation as yet. However, it may be caused by the serine

to glycine alteration of amino acid 264 on the D1 protein of PS II. The electro-negative serine hydroxyl group may be the reason that the quinone complex is difficult to reduce twice in a very short time, which actually happens when a RC receives two light quanta during a single flash. The presence of an uncharged amino acid residue in the mutants might be held responsible for less net negative charge on Q_B.

Our model was used to analyze data published by Delrieu (1983). A fit assuming the miss parameters α_0 and α_2 to be 0 resulted in a sum of the quadratical differences of 0.6 % or less, which is at least as well as the originally published fits using unequal miss parameters.

Table 3.4. Fit of published data of other authors using our model. Columns 1, 3 and 5 give the originally published values, columns 2, 4 and 6 show values calculated with our model, for experiments published (Delrieu 1983). $Ec=\sqrt{(\Sigma_{1\text{-}10}(Y_{EXP}-Y_{TH})^2)}$ *(in %). nda = no data available.*

	Exp.1		Exp.2		Exp.3	
S_0	nda	0.10	nda	0.06	nda	0.135
β	0	0	0	0.01	0	0.02
α^0	0.018	0	0.01	0	0	0
α_1	0.018	0.13	0.01	0.36	0	0.31
α_2	0.63	0	0.61	0	0.542	0
α_3	0.018	0.58	0.01	0.385	0	0.33
Ec	0.58	0.57	0.76	0.51	0.51	0.52

The values for the double hit parameter are very low. A possible explanation is the use of a flash with a very short duration. After dark adaptation for 5 min after 20 preflashes, as performed by Delrieu, still some 10 % of the OEC's is in state S_0. In our experiments this percentage is 0, due to prolonged dark adaptation. This fact is not corrected for in the

Delrieu-model. These fits indicate that the model should be valid for experiments carried out in different conditions, using different equipment.

In conclusion, the model presented here can be used to describe the flash-induced oxygen evolution adequately. Accurate determination of the chloroplast- and equipment-dependent parameters D, S_0, α_n and β is required in order to use them in future calculations of the exchange parameters of herbicides on the Q_B binding site. The method for calculating these parameters has been described before (Vermaas 1984, Naber and Van Rensen 1987).

4 Exchange of herbicides at the quinone binding site

4.1 Introduction

Many herbicides acting on photosynthesis block the photosynthetic electron transport chain. The inhibition of electron flow is caused by preventing the oxidation of the primary quinone electron acceptor Q_A of photosystem II (PS II) by the secondary acceptor quinone Q_B. The herbicides which inhibit at the PS II site compete with Q_B for binding. Most of the effective compounds show a structural resemblance to the quinone. In this Chapter a description is given of a method to determine the exchange rates of herbicides at the quinone binding site.

4.1.1 Flash-induced oxygen evolution

The method for measuring herbicide binding kinetics is based on comparison of experimental and calculated flash-induced oxygen evolution patterns of isolated broken chloroplasts. It was introduced by Vermaas et al. (1984b). The oxygen release pattern shows a periodicity of four, with a maximum on flash 3 (Joliot et al. 1971). Damping of this pattern is thought to be caused mainly by the occurrence of misses (α) and double hits (β) of reaction centers by the excitation light (Kok et al. 1970). In Chapter 3, a modification of the original Kok model was made, assuming a dependency of α on the redox states at the PS II donor- and acceptor-sides. The values for α and β were derived from control experiments without herbicides. The calculation of these system parameters is described in Chapter 3. The values of the parameters are assumed to be determined mainly by the experimental conditions, e.g. light intensity, flash duration and chloroplast concentration. The changes in the amount of oxygen

evolution and the patterns, caused by incubation with a herbicide, are caused by a blocking of electron transport.

4.1.2 Herbicide exchange parameters

Compounds from different chemical classes, e.g. urea, triazines and cyanoacrylates, bind to the same region of the D1 protein (Pfister and Arntzen 1979, Mattoo et al. 1981, Gardner 1981 and 1989, Trebst 1986). This binding region is situated at the acceptor side of PS II. It is part of a binding environment for two different plastoquinone molecules, the tightly bound primary acceptor Q_A and the easier exchangeable secondary acceptor Q_B (Velthuys 1981, Wraight 1981). Q_B can be reduced by Q_A to Q_B^{2-}, which subsequently is protonated to Q_BH_2. Both the oxidized and the fully reduced form are rapidly ($k = 50$ to $100\ s^{-1}$) exchanged for oxidized plastoquinone molecules from the PQ-pool, but the semi-quinone form is not (Velthuys 1981, Wraight 1981).

A PS II-herbicide, if present, can competitively inhibit binding of a quinone molecule by occupying the same or a nearby located binding environment (Tischer and Strotmann 1977, Pfister and Arntzen 1979, Reimer et al. 1979, Pallet and Dodge 1979, Moreland 1980, Velthuys 1981, Jursinic and Stemler 1983, Kyle 1985, Trebst 1987, Van Rensen 1989, Gardner 1989). An open, but inhibitor-occupied reaction center can make only one turnover, resulting in the reduction of the acceptor side complex, because the electron transfer towards the PQ-pool is blocked. This can easily be monitored by measuring fluorescence induction, as is described in Chapter 5.

Most herbicides exchange with the native plastoquinone at the binding site on a second timescale. This can be monitored with a Clark electrode. A few seconds after herbicide addition, inhibition of the oxygen evolution can be measured. When chloroplasts are illuminated with flashes, the herbicides have a certain probability to replace a quinone molecule during the flash train. When the exchange rate is low, i.e. when the average residence time of the herbicide molecules on D1 is much longer than the duration of the flash train of 10 flashes, almost no exchange

takes place in this time. The result is a lower net oxygen production, with the same oscillation patterns as the control without herbicide. Reaction centers with a bound herbicide only make one turnover, and produce no oxygen, whereas the other centers behave like the control. When the herbicide molecules are exchanged at a rate which is of the same order of magnitude as the flash frequency, changes are observed in the pattern. This is the result of reaction centers getting out of phase with each other. During the flash train a herbicide molecule can block electron transport in a particular chain, thus blocking PS II oxygen evolution. On the other hand a herbicide can be released from a center, which then starts evolving oxygen at a random flash as compared to the bulk of uninhibited centers. This results in a faster damping in the oscillation. By varying the flash frequency between 10 Hz and 0.25 Hz, differently damped patterns can be observed for herbicides with different exchange rates.

The exchange parameters are obtained by fitting experimental data to those calculated with a kinetic model. This model is derived from equations 4.1 and 4.2.

In these equations, S_n (n = 0,1,2,3) represents the redox state of the OEC. In thoroughly dark-adapted thylakoids, nearly 100 % of the centers are in the stable S_1-state (Chapter 3, Vermaas 1984a).

$$S_n{\cdot}(Q_A{\cdot}Q_B) + I \underset{E_2}{\overset{E_1}{\rightleftharpoons}} S_n{\cdot}(Q_A{\cdot}Q_B) + Q_B \qquad (4.1)$$

$$S_n{\cdot}(Q_A{\cdot}Q_B)^- + I \underset{E_4}{\overset{E_3}{\rightleftharpoons}} S_n{\cdot}(Q_A{\cdot}Q_B)^- + Q_B \qquad (4.2)$$

The E_1 and E_2 parameters are dependent on the concentration of free inhibitor. However, under dark-adapted conditions in which the PQ-pool is oxidized, E_3 and E_4 are not influenced by the PQ-concentration. This is caused by the fact that E_3 and E_4 are determined rather by the release of

the inhibitor from its binding environment than by the binding of a PQ-molecule, since the electron transport from Q_A to the PQ-pool has a half-time significantly less than 5 ms. (Robinson et al. 1984). This is much faster than the inhibitor exchange.

In the experiments, inhibitor concentrations were chosen around the I_{50}-value. Thus the amplitude of the signal still permits accurate measurements, while at the same time a reasonable damping can be observed.

4.1.3 Properties of herbicides used

Herbicides from several classes were used in this study. They all bind to the PS II acceptor side. At least some of these classes appear to share the same binding niche (Pfister and Arntzen 1979, Kyle 1985, Gardner 1989), but the exact binding environments are proposed not to be identical (Trebst 1987, Van Rensen 1989). The triazines, urea and cyanoacrylates are proposed to bind close to serine-264, which is located at the stroma side of the D1 protein. Phenolic compounds display an interaction with the histidine-215. This histidine is located within a membrane spanning helix rather than at the membrane surface.

Atrazine is a triazine compound. DCMU is a widely used representative of the urea-type herbicides, and ioxynil is a phenolic compound. The JNP-chemicals are cyanoacrylates, which are as yet not commercially used. They provide interesting possibilities for research, since several stereo-isomers are available. Although chemically almost identical, these show a significant difference in inhibition of flash-induced oxygen production by isolated chloroplasts. Many QSAR investigations have been performed with this class of compounds (Phillips 1988, Phillips and Huppatz 1984 and 1987, Huppatz and Phillips 1984 and 1987a, b and c).

4.2 Results and discussion

Chenopodium album chloroplasts isolated from a wildtype were compared with those from a triazine-resistant biotype. The exchange parameters E_1 to E_4 were calculated for different types of herbicides. Pea chloroplasts were used as reference material.

4.2.1 The situation at the photosystem II acceptor side

The herbicide exchange parameters were calculated using the inhibitor exchange model described by Vermaas (1984) with modifications as described in Chapter 3. Different values of the miss parameters α were assumed to be correlated to the four different S-states. The α-parameters were chosen to be high when a reduced acceptor complex was present, and zero in case of an oxidized complex. However, in the presence of a herbicide, no good fit could be obtained between experimental oxygen productions and the calculated values.

In Chapter 3, a semi-reduced state for the PS II acceptor complex, $(Q_A.Q_B)^{-\cdot}$ was assumed to exist after dark incubation. This state was supposed to be formed in the absence of herbicide (see Chapters 3 and 6). The consequence is that in the presence of a herbicide a permutation in the values of the α-parameters had to be assumed, compared to those calculated in Chapter 3. If this was done, the agreement between theoretical and experimental data was much better. In contrast to the situation as described in Chapter 3, a fully oxidized complex seemed to exist after long dark adaptation with a herbicide. The cause may be that the inhibitor molecule destabilizes an electron present on Q_A. The occupation of the Q_B binding site by the inhibitor prevents stabilization of an electron over two different quinone molecules. As a consequence the miss parameters which were used for calculation of the exchange parameters were shifted one step compared with the values of Chapter 3. This means that those on the first and other odd flashes (α_1 and α_3) become zero, while α_0 and α_2 have values corresponding to the non-zero values as listed in Table 3.2.

4.2.2 The exchange parameters

The parameters E_1 and E_3 represent the exchange rates of herbicides to an oxidized binding environment. They are supposed to be a factor of 10 to 100 higher than the corresponding parameters for a reduced complex. This is caused by the fact that the secondary acceptor Q_B binds very strongly to its binding niche when it is in the semi-quinone form, whereas both the fully reduced and the oxidized forms are easily exchanged (Velthuys 1981, Wraight 1981). In the semi-reduced state it is then difficult to replace the quinone by a herbicide molecule. Addition of DCMU to a semi-reduced complex $(Q_A.Q_B)^-$ was shown to induce a reverse electron flow from Q_B^- towards Q_A (Velthuys and Amesz 1974, Jursinic and Stemler 1983). When a herbicide is bound, an electron on the quinone complex is poorly stabilized, at least at Q_A (Van Gorkom et al. 1975). This can be monitored by measuring decay of fluorescence intensity after illumination with a single saturating flash in the presence of DCMU (Robinson and Crofts 1983, Ooms, pers. comm.) or by measuring herbicide binding as a function of the preillumination flash number (Wollman 1978, Laasch et al. 1983). The relaxation of $Q_A^-.I$ to $Q_A.I$ appears from our experiments to be more rapid than was suggested before (Laasch et al. 1983, Velthuys and Amesz 1974).

A bound herbicide destabilizes a negative charge at the acceptor complex. Mutually, the presence of an electron on Q_A accelerates the release of the herbicide from its binding site. This provides an obvious explanation for the observation that the release kinetics of the herbicides are slower when a fully oxidized complex exists, which is reflected in the fact that E_4 is found to be higher than E_2 in many experiments. For all herbicides tested E_1 was significantly higher than E_2. The effectiveness of a herbicide is thus mainly determined by the possibility of binding to an oxidized complex.

Atrazine proved to bind faster than DCMU, but it is also released more easily. In the resistant biotype of *Chenopodium*, the atrazine binding constant E_1 is slightly decreased as compared to the wildtype. However, the resistance seems to originate from a significant increase in the release kinetics, which is reflected in higher values of the parameter E_3. This can

be explained on the molecular level. The "on" kinetics of a compound to the binding environment are determined principally by the accessibility of the niche to the compound. This is determined by the chemical structure of the herbicide, especially its molecular dimensions, charges and hydrophobicity. These properties are, of course, the same when atrazine is added to resistant or to sensitive chloroplasts. A very slight change of hydrophobicity of the binding pocket can be expected as a result of the serine to glycine substitution (see Chapter 5). However, the atrazine molecule cannot be stabilized in its binding environment in the mutant protein, probably because the ser-OH group provides an important H-bonding possibility in the wildtype. The result is a decrease in herbicidal activity of 2 to 3 orders of magnitude (Table 4.4). In the case of ioxynil the situation is reversed, though the difference in activity in both biotypes is far less as compared with atrazine. With ioxynil only a slight difference in binding to the D1 protein is observed, but now the release from the resistant biotype is 10-fold slower than from the wildtype protein. The hydroxyl group of ser-264 apparently destabilizes binding of ioxynil. This is not in accordance with the model of Trebst (1986), who proposed that phenolic inhibitors would bind to the his-215.

The cyanoacrylate compounds used are two pairs of stereo isomers: JNP-1166 and JNP-1174, and JNP-1222 and JNP-1223. The isomers differ only in the orientation of the atoms around the indicated carbon atoms (see Chapter 1 for structure formulas), and are the same with regard to physico-chemical properties as e.g. hydrophobicity. Therefore the E_1-parameters are expected to be more or less the same within one pair of herbicides, and different inhibition properties are presumed to originate from dissimilar release behavior. This was observed for the compounds JNP-1166 and JNP-1174, and to a lesser extent in case of JNP-1222 and JNP-1223. In the latter compounds, which do not inhibit electron transport as strong as JNP-1166 and JNP-1174, the large side groups of the chiral carbon atom could be interfering with binding. In Chapter 6 a model concerning binding of these compounds is discussed. The differences in inhibition properties are found to originate mainly from differences in the E_3 values. In both pairs of compounds the asymmetrical carbon atom

Table 4.1a. Values for the exchange parameters measured in pea chloroplasts.

	$E_1(\mu M^{-1}.s^{-1})$	$E_2(\mu M^{-1}.s^{-1})$	$E_3(s^{-1})$	$E_4(s^{-1})$
Atrazine	0.24	0.02	0.005	0.004
DCMU	0.01	0.001	0.013	1.0
Ioxynil	0.1	0.01	0.03	1.0
JNP1166	0.5	0.02	0.04	0.003
JNP1174	0.4	0.001	1.0	2.0
JNP1222	0.015	0.0002	0.1	0.01
JNP1223	0.002	0.0001	0.09	0.15

Table 4.1b. Values for the exchange parameters measured in Chenopodium S chloroplasts.

	$E_1(\mu M^{-1}.s^{-1})$	$E_2(\mu M^{-1}.s^{-1})$	$E_3(s^{-1})$	$E_4(s^{-}c^{1})$
Atrazine	0.1	0.03	0.11	0.04
DCMU	0.056	0.0015	0.008	0.0035
Ioxynil	0.4	0.02	2.0	2.0
JNP1166	0.1	0.02	0.013	0.05
JNP1174	0.15	0.0012	0.67	0.1
JNP1222	0.01	0.0004	0.1	0.05
JNP1223	0.18	0.002	1.0	0.005

Table 4.1c. Values for the exchange parameters measured in Chenopodium R chloroplasts.

	$E_1(\mu M^{-1}.s^{-1})$	$E_2(\mu M^{-1}.s^{-1})$	$E_3(s^{-1})$	$E_4(s^{-1})$
Atrazine	0.05	0.002	15	2.25
DCMU	0.064	0.002	0.001	0.001
Ioxynil	0.22	0.04	0.2	0.05
JNP1166	0.02	0.004	0.002	0.02
JNP1174	0.02	0.0012	0.1	0.05
JNP1222	0.025	0.02	2.0	1.0
JNP1223	0.025	0.012	0.09	0.0045

is located at a different position relative to the main functional group for herbicidal activity, the amino-acrylate group. This fact does not seem to have a clear influence on the functioning of the compounds when compared with each other in susceptible and resistant chloroplasts. In Chapter 6 a model is proposed in which the asymmetrical carbon is located more or less in the same position relative to the binding niche in all 4 cyanoacrylates used.

4.2.3 The inhibition coefficients

The E_1 and E_3 values can be used for an estimation of the I_{50}-concentrations of the applied herbicides. Since the exchange is relatively low in case a semi-reduced acceptor complex exists, E_2 and E_4 are not included in the calculations. From Eqn. (4.1) it can be derived that

$$d([S_n.Q_A.Q_B])/d(t)=E_3[S_n.Q_A.I]-E_1([S_n.Q_A.Q_B][I]) \quad (4.3)$$

In a steady-state situation the concentration of $S_n.Q_A.Q_B$ is constant, and thus

$$E_3/E_1 = [S_n.Q_A.Q_B][I]/[S_n.Q_A.I] \quad (4.4)$$

When 50 % of the reaction centers are inhibited, the concentrations of $S_n.Q_A.Q_B$ and $S_n.Q_A.I$ are equal to one another, and thus

$$E_3/E_1=[I_{50}] \quad (4.5)$$

where $[I_{50}]$ stands for the I_{50}-value in μM. The data in Table 4.1 are used for a calculation of the I_{50}-values of the herbicides in different chloroplasts. Those calculated data are compared to the pI_{50}-values as measured with a Clark-type of oxygen electrode under continuous illumination.

Table 4.2. Measurement and calculation of pI_{50}-values for the herbicides used.

	Pea		Chenopodium S		Chenopodium R	
atrazine	7.0	7.7	6.5	6.0	< 4	3.5
DCMU	7.5	5.9	7.5	6.8	6.7	7.8
ioxynil	6.5	6.5	6.6	5.3	7.0	6.0
JNP1166	7.8*	7.1	7.5	6.9	7.3	7.0
JNP1174	5.8*	5.6	6.0	5.4	6.3	5.3
JNP1222	5.4*	5.2	5.6	5.0	5.0	4.1
JNP1223	6.0*	4.3	5.7	5.2	5.0	5.4

The left columns show pI_{50}-values measured with a Clark-type of electrode under continuous illumination, the right columns give values of E_3/E_1. Values are given as the negative log of the molar concentration.
** : data obtained from Dr. J. Phillips (pers. comm.).*

The values calculated from data obtained by fitting flash-induced oxygen productions are in almost all cases lower than those measured directly in a standard Clark electrode. This can be explained as the result of a difference in measuring method. Under continuous illumination the PS II acceptor complex is predominantly in a reduced state. This significantly slows down exchange of bound herbicides with plastoquinone molecules. Reaction centers which are blocked during dark adaptation before starting the measurement (necessary for binding of the herbicide to D1) do not have a chance to get rid of the inhibitor. This is reflected in the low value for the E_4-parameter. When chloroplasts are illuminated with short flashes, spaced seconds apart, a certain (small) amount of herbicide can be replaced with PQ. These centers might thus be able to transport an electron to the secondary acceptor in the dark period between the flashes, and start producing oxygen again on the next flash. The dark period is also long enough for a certain fraction of the reduced complexes $Q_A^-.I$ to dissipate the negative charge, since it is an instable state (Velthuys and Amesz 1974, Laasch et al. 1983). Thus, on the next flash, the acceptor

can be reduced again. This may result in a higher oxygen production than would be expected on the basis of the number of reaction centers containing a bound inhibitor molecule.

5 Characterization of the triazine-resistant biotype of *Chenopodium album*

5.1 Introduction

In this chapter the characterization of the *Chenopodium album* biotype resistant to triazine herbicides is described. Resistant and sensitive biotypes cannot be distinguished visually. On the whole-plant level the most simple method to discriminate between wildtype and resistant biotype is spraying the plants with a solution of 0.1 mM atrazine. The wildtype plants will show serious damage within 48 hours and die within a week, whereas the resistant biotype does not visibly suffer from any influence of the herbicide. More sophisticated is the measurement of fluorescence induction curves, a method which can be applied to intact leaves or to isolated chloroplasts. Isolated thylakoids can also be used for oxygen evolution measurements. Addition of atrazine to the reaction medium in concentrations up to 0.1 mM will not severely inhibit the oxygen production in the resistant biotype, whereas in the wildtype the inhibition is complete. The most laborious method, which does not yield any information concerning the level of resistance, is sequence analysis of the gene responsible for the change from wild type to resistant biotype. Effects of atrazine on electron flow were assayed routinely. In this Chapter, results are presented about fluorescence induction measurements and sequence analyses.

5.2 Herbicide binding to photosystem II

The part of the D1 protein where the quinone acceptor Q_B and many photosynthesis inhibitors bind is described in this paragraph. Changes in the gene coding for the D1 protein are responsible for the resistance to triazine compounds in higher plants.

5.2.1 The D1 protein and its environment

In the photosynthetic electron transport chain of green plants and algae a 32 kD MW protein plays an important role. On the basis of electrophoretic patterns this protein was indicated as D1 protein (Bottomley et al. 1974, Ellis 1977, Chua and Gillham 1977). It was later shown to be identical to the herbicide binding protein, which provides the binding environment for many PS II herbicides (Pfister and Arntzen 1979, Mattoo et al. 1981, Gardner 1981). Recently the protein was shown to constitute part of the photochemical reaction center of the photosystem II core complex, together with the structurally related D2 protein and the cytochrome b_{559} complex (Deisenhofer et al. 1984, Trebst 1986, Nanba and Satoh 1987). D2 is the protein which carries the primary PS II quinone electron acceptor Q_A, while D1 binds the secondary acceptor Q_B (Velthuys and Amesz 1974, Arntzen et al. 1982). Between Q_A and Q_B a non-heme iron atom is located (Ikegami and Katoh 1973, Petrouleas and Diner 1986). This iron is proposed to play a role in the binding of bicarbonate (Van Rensen et al. 1988, Nugent et al. 1988). Both proteins are proposed to form 5 hydrophobic, thylakoid membrane spanning α-helices (Trebst 1986). They are located opposite to each other, close enough for many interactions on the molecular level. They form a more or less rigid structure, modeled in the ideal position for transport of electrons from the primary donor Z to the final PS II acceptor Q_B.

Structure analysis with 3 Å resolution showed only slight differences between the D1 and D2 proteins. This analysis suggested e.g. that only the tyrosine residue Z of D2 and not tyrosine D, in the equivalent position of D1, was the primary donor to the reaction center (Deisenhofer et al. 1985). In very elegant site-directed mutagenesis experiments this hypothesis was verified (Debus et al. 1988). The binding sites for the primary and secondary acceptors Q_A and Q_B also show a large degree of homology. Nevertheless, Q_A is tightly bound and not readily exchanged with natural or artificial molecules in the membrane environment.

The other quinone Q_B is continuously being replaced in the light. Upon reduction plastoquinol (Q_BH_2) is formed, which is subsequently exchanged with an oxidized plastoquinone (Q_B) from the plastoquinone

pool in the membrane. Here the quinol is reoxidized via the cytochrome b_6/f complex, thus transporting electrons towards PS I. Finally, $NADP^+$ is reduced. In this redox cycle the protons are taken up from the chloroplast stroma, and transported across the thylakoid membrane into the lumen. A proton gradient is built up across the thylakoid membrane, enabling the ATPase complex to phosphorylate ADP to the high-energetic ATP (Mitchell 1961). If for some reason electron transport from PS II to PS I is no longer possible, no more NADPH and ATP can be synthesized. This eventually leads to the death of the plant cells.

Triazine herbicides inhibit photosynthesis by blocking the PS II associated electron transport from the reaction center to the plastoquinone pool (Arntzen et al. 1979, Moreland 1980). The mode of action of these herbicides is their ability to prevent binding of Q_B, by either occupying or blocking its binding site (Kyle 1985), or by inducing a conformational change in the target D1 protein (Van Rensen 1989). The hydrophilic amino acid residues of this protein connecting helices 4 and 5 are located at the stroma side of the membrane (Trebst 1987). This is the environment where the quinone acceptors and the triazine and urea type herbicides bind. Resistance to triazine compounds was shown to be the result of an alteration in the D1 protein (Pfister and Arntzen 1979, Pfister et al. 1981). This modification is thought to result in a change of the binding site, influencing herbicide binding much stronger than quinone binding.

5.2.2 The psbA gene

Like about half of the genes coding for thylakoid membrane proteins, the psbA gene for the D1 protein is located on the chloroplast genome (Bedbrook et al. 1978, Mattoo et al. 1981, Steinback et al. 1981, Shinozaki et al. 1988). The chloroplast genome was shown to consist of a single covalently closed circular molecule, occurring in 10 to several hundreds of identical copies per chloroplast. The complete base sequence was determined for *Marchantia polymorpha* (Ohyama et al. 1986) and for *Nicotiana tabacum* (Shinozaki et al. 1988), revealing a 155 kb sequence containing a 25 kb inverted repeat.

In *Chlamydomonas reinhardii* the psbA gene is located within the inverted repeat and thus exists in 2 copies per genome (Erickson et al. 1984a), whereas in higher plants only a single copy of the gene has been found (Bedbrook et al. 1978, Zurawski et al. 1982, Spielmann and Stutz 1983, Sugita and Sugiura 1984). In *Synechocystis* 6803 the psbA gene belongs to a three-membered multigene family (Jansson et al. 1987). The higher plant genes do not contain introns, but in algae four introns were found (Erickson et al. 1984a). These introns can complicate DNA sequence analysis of a gene, because they are non-coding sequences.

The D1 proteins isolated from different plants show a high degree of homology. The nucleotide sequence can differ slightly, but most mutations turn out to be silent (Erickson et al. 1985a). For *Spinacia oleracea*, *Nicotiana tabacum* and *N. debneyi* the primary structures of the coded proteins are identical (Zurawski et al. 1982, Sugita and Sugiura 1984), while the D1 amino acid sequence of *Glycine max* and *Sinapsis alba* show 2 resp. 3 deviations near the C-terminal part (Spielmann and Stutz 1983, Link and Langridge 1984).

The psbA gene product is highly homologous between higher plants or algae and photosynthetic bacteria, even though the bacterial equivalent of the D1 protein, i.e. the L-subunit, is somewhat smaller. In fact this homology was among the first observations suggesting the function of D1 in plant reaction centers, since the L-subunit had been identified earlier. With regard to the structure of the Q_B binding niche, the ser-223 residue of the L-subunit in photosynthetic bacteria is equivalent to the D1 ser-264 of higher plants. The serine forms a H-bond to the acceptor Q_B, whereas ile at position 229 is in Van der Waals interaction with the quinone. Together with these residues, also phe-216 is involved in herbicide binding. In higher plants the equivalent of phe-216 is found at position 255.

5.2.3 Mutations in the psbA gene in different organisms

Since 1968, different biotypes of many plant species showing resistance towards electron transport inhibitors have been found.

Especially in areas where intensive spraying with herbicides has occurred, appearance of resistant weeds began to form a practical problem (Ryan 1970, Arntzen et al. 1979). The first herbicide-resistant plant to be characterized on the DNA level was a triazine-resistant biotype of *Amaranthus hybridus* (Hirschberg and McIntosh 1983). The authors sequenced the complete psbA gene, and discovered a single base transition causing a serine to glycine mutation in the D1 protein. Since then mutations in the D1 or L proteins of several higher plants, algae and bacteria have been demonstrated (Hirschberg et al. 1984, Goloubinoff et al. 1984, Reith and Strauss 1986, Erickson et al. 1984b, Erickson et al. 1985b, Golden and Haselkorn 1985, Johanningmeier et al. 1987, Rochaix and Erickson 1988, Barros and Dyer 1988, Gilbert et al. 1985, Paddock et al. 1988). An overview of the most important mutations described so far is given in Table 5.1.

5.3 A simple procedure for DNA and mRNA sequence analysis

DNA sequence analysis usually is performed on isolated, purified and then cloned DNA fragments, using either the Maxam and Gilbert chemical method or the dideoxy method of Sänger (Sänger et al. 1977). These methods are laborious and time-consuming. In this Chapter a procedure is described which enables simultaneous isolation of largely intact total DNA and RNA. These can be used directly to determine partial sequences of the psbA gene or the corresponding mRNA coding for the D1 protein. This method avoids identification, isolation and cloning of the fragments.

Table 5.1. Mutations in the psbA gene product causing herbicide-resistances in photosynthetic organisms.

Organism	Mutation	Res.	Reference
D1 protein resp. L-subunit isolated from:			
A. hybridus	264 ser-gly	atra	Hirschberg & McIntosh 1983
S. nigrum	264 ser-gly	atra	Hirschberg et al. 1984
S. nigrum	264 ser-gly	atra	Goloubinoff et al. 1984
B. napus	264 ser-gly	atra	Reith & Strauss 1987
P. annua	264 ser-gly	atra	Barros & Dyer 1988
C. album	264 ser-gly	atra	Bettini et al. 1988
C. album	264 ser-gly	atra	This thesis
N. tabacum	264 ser-thr	atra	Sato et al. 1988
N. plumbaginifolia	264 ser-asn	terb	Páy et al. 1988
An. nidulans	264 ser-ala	DCMU	Golden & Haselkorn 1985
Chl. reinhardii	264 ser-ala	atra	Erickson et al. 1984b
Chl. reinhardii	219 val-ile	DCMU	Erickson et al. 1985b
Chl. reinhardii	255 phe-tyr	atra	Erickson et al. 1985b
Chl. reinhardii	251 ala-val	atra	Johanningmeier et al. 1987
Chl. reinhardii	256 gly-asp	atra	Rochaix & Erickson 1988
Chl. reinhardii	275 leu-phe	DCMU	Rochaix & Erickson 1988
E. gracilis	264 ser-thr	DCMU	Johanningmeier et al. 1989
Syn. 7002	211 phe-ser	atra	Buzby et al. 1987
Syn. 7002	219 val-ile	DCMU	Gingrich et al. 1987
Syn. 7942	255 phe-tyr	atra	Brusslan & Haselkorn 1988
Syn. 7942	264 ser-ala	atra	Golden & Haselkorn 1985
Rps. sphaeroides	229 ile-met	atra	Gilbert et al. 1985
Rb. sphaeroides	222 tyr-gly	terb	Paddock et al. 1988
Rb. sphaeroides	223 ser-pro	terb	Paddock et al. 1988
Rb. sphaeroides	229 ile-met	terb	Paddock et al. 1988

atra = atrazine, DCMU = diuron, brom = bromacil, terb = terbutryne. A=Amaranthus, S=Solanum, B=Brassica, P=Poa, C=Chenopodium, N=Nicotiana, An=Anacystis, Chl. = Chlamydomonas, E=Euglena, Syn=Synechococcus, Rps. = Rhodopseudomonas, Rb. = Rhodobacter.

5.3.1 Principles of nucleic acid sequencing

Determination of the nucleotide sequence of a fragment of DNA or RNA generally requires cloning into a plasmid or bacteriophage. The next step is transformation of a bacterium, followed by amplification and isolation of the vector. A primer complementary to a vector sequence can then be used to initialize synthesis of the complementary DNA strand. In the method described in this thesis a specially designed and chemically synthesized oligonucleotide, which hybridizes to the isolated DNA-fragment, is used as a primer.

In both procedures, the complementary strand is synthesized in the presence of deoxynucleotide-triphosphates, combined with any of 4 dideoxynucleotide-triphosphates. These lack the 3'-OH group, which is the group binding the next deoxynucleotide. Thus, when such a dideoxynucleotide is built into the growing chain, the elongation stops. By choosing the ratio of dideoxy- to deoxynucleotides optimally, the newly synthesized strands can vary in length from a few to many hundreds of bases. The synthesized fragments in the 4 reaction mixtures are subsequently separated according to their sizes on polyacrylamide-urea gels. The length of each fragment can be determined relative to the primer. Since the last base of the template strand must be complementary to the dideoxynucleotide that was present in that particular mixture, the sequence of the DNA can be read from the 4 lanes on the gel. The reactions are schematically represented in Fig. 5.1.

5.3.2 Method for characterization of psbA-mutations

A very fast and simple procedure is used to perform a direct partial DNA or mRNA sequence analysis of a gene. A prerequisite is that the sequence of a homologous gene or that of a small flanking region of the DNA must be known, because a synthetic probe, about 15 to 20 nucleotides in length, must be hybridized to the DNA. This DNA functions as the recognition site for DNA-polymerase, and as the starting point for synthesis of a complementary strand. The more selectivity the probe

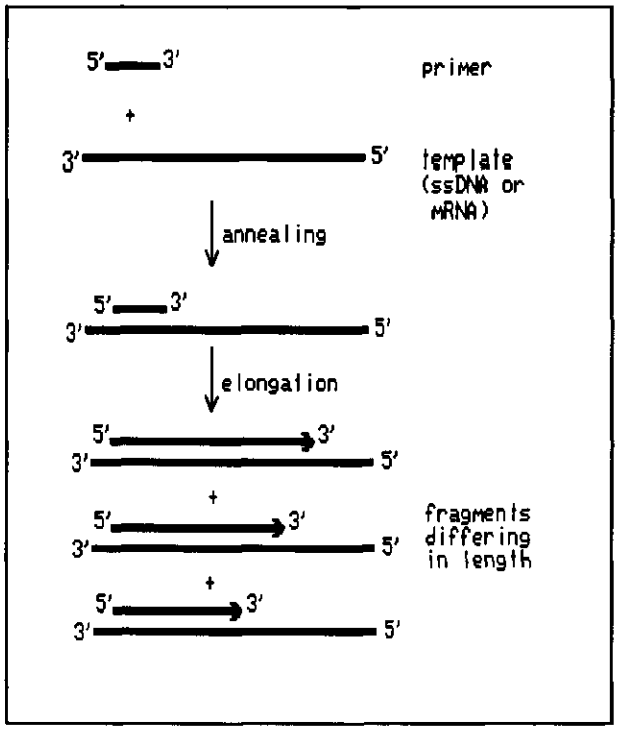


Fig. 5.1. Reactions for DNA or RNA sequence analysis. A selective primer is annealed to the template, and elongated by a DNA-polymerase. The chain elongation is stopped by dideoxy nucleotide incorporation, resulting in a gamma of fragments with different lengths. These can by separated on PAA-gels.

displays, the better the desired fragment can be recognized in the isolated plant DNA. In case a mutation in a certain fragment of a gene is expected, a sequence complementary to the (wildtype) DNA is a very suitable probe. The probe must hybridize about 20-100 bases towards the 3'-end of the coding strand relative to the sites where the mutation is expected. The newly synthesized strand, which of course is elongated in 5' to 3' direction, then contains the region with the mutation close to the end of the primer. This assures easy reading of the sequence on the gels, because shorter fragments are both synthesized and separated easier than longer ones.

The advantage of the method is that time-consuming isolation of DNA, fragment purification and cloning are not required. When no specific primer is available, these steps are necessary to obtain a larger amount of the particular fragment. The standard plasmid cloning system must then be used in combination with a primer hybridizing to the plasmid.

When sequences from homologous genes in other plants or bacteria are known, any region showing a high degree of conservation can potentially be used to design a specific but uniform probe for use in different species. When slight differences in genes are expected, a mixed probe can be used. This is a mixture of nearly identical sequences. The method is very suitable to test for a mutation in the psbA gene. Only a small part of the gene, containing the known sites of mutation, has to be sequenced. This fragment can, if necessary, be amplified using the PCR (polymerase chain reaction) technique. In this case one (or both) of the primers needed for the amplification can also be used in the sequencing reactions. Either single stranded DNA or mRNA can be used as a template for synthesis of a new DNA strand. The main distinction between these procedures is the use of different types of DNA-polymerizing enzyme.

The D1 protein is one of the most abundant chloroplast proteins. It shows a high rate of turnover in the light (Mattoo et al. 1981, Wettern 1986). A large amount of psbA-mRNA is present in light grown plants (Lönneberg et al. 1988). A high content of the mRNA-fragment to be sequenced enhances specific hybridization, thereby eventually raising the strength of the radioactive signal on the sequencing gels.

The psbA nucleotide sequence shows large homologies between different species. This allows the use in many photosynthetic organisms of a selective synthetic probe as a universal primer. When the sequence of the wildtype-psbA gene is unknown, a primer can be used which is complementary to a sequence of a homologous gene, preferably from a related species. In our case, 2 out of 3 probes originally designed for *Chlamydomonas reinhardii* (Johanningmeier et al. 1987) proved to hybridize well to total RNA from *Chenopodium*. When a total RNA preparation is made, a selective probe can be used as a primer for the reverse transcriptase, which synthesizes the complementary DNA strand from a RNA template. The probe can be 5'-labeled with a [γ-^{32}P]ATP, allowing the synthesis to be performed without using any further radioactive labels. However, the most important advantage is a signal of constant intensity, independent of the length of the synthesized fragment.

The binding environment on the D1 protein for many herbicides is the region between the membrane spanning helices IV and V (Trebst 1987).

This part of the protein, globally between amino acid residues 220 and 280, is the region where all mutations causing herbicide-resistance investigated until now are located (Table 5.1, Brusslan and Haselkorn 1988, Rochaix and Erickson 1988). For the analysis of a triazine- or DCMU-resistant biotype, it seems justified to assume that the mutation is located within this fragment of D1. Thus, it is unnecessary to determine the complete psbA DNA sequence. Using one single primer, a stretch of at least 200 nucleotides should easily be read on a single gel.

5.3.3 Application of the method

A detailed description of the nucleic acid isolation procedure and the sequencing reactions is given in Chapter 2. For the strand synthesis 2 µl AMV reverse transcriptase (15 u/µl, Pharmacia) was used in combination with 50 µg total RNA as a template. For DNA sequence analysis about 25 µg nucleic acid was used as a template for standard strand synthesis by T7 DNA polymerase. In both types of reactions $5*10^6$ cpm (5'-^{32}P)-labeled synthetic primer was used. This 17-mer oligonucleotide, used as a mixed probe of 4 different strands, has a base sequence 5' GC$^{A}/_{T}$GT$^{G}/_{A}$AACCAAATACC 3'. It is complementary to the non-coding DNA strand (and thus to the mRNA) in the region from codon 282 to 287. Fragments were separated on 8 % polyacrylamide-urea sequencing gels, electrophoresed at 70 W. After running, gels were exposed to Kodak XA X-ray film using an intensifying screen at -80 °C.

5.4 Chlorophyll *a* fluorescence induction measurements

Chlorophyll *a* fluorescence induction measurements can be used for an easy estimation of the resistance of plants to herbicides. Fluorescence is the emission of energy, which is not used for photochemical and other dissipative processes. When electron transport is not inhibited, and the acceptor side of photosystem II is oxidized by electron extraction by PS I, no negative charge is present on the primary acceptor Q_A of PS II.

Oxidized Q_A quenches the chlorophyll *a* fluorescence (Duysens and Sweers 1963). Thus, after dark adaptation in the absence of herbicides the induction of fluorescence of PS II is slow, because a large electron-accepting quinone pool must be filled before Q_A is fully reduced. At the light intensity used, the time before the maximum fluorescence level is reached is 1 to 2 s. A large number of light quanta is used to induce stable charge separations. When a herbicide is present which blocks the electron transport between Q_A and the PQ-pool, like most photosynthesis inhibitors do, a negative charge on Q_A prevents another charge separation in the reaction center. The absorbed energy is then dissipated in the form of emitted light, fluorescence. This is reflected in a much faster rise of the fluorescence curve, reaching the maximum fluorescence level within 0.5 s after switching on the actinic light at the intensity used.

The presence of negative charges on the PS II acceptor side can be determined quantitatively by measuring the area over the induction curve. The area is larger when the rise in fluorescence is slower. This is the case when the acceptors, Q_A and Q_B, and the quinone pool are mainly oxidized, and electrons can be transported towards cyt b/f and PS I easily. When electron flow is inhibited, the excitation light cannot be used to induce a charge separation and is emitted as fluorescence. The rise in fluorescence is fast, and the area over the curve thus small.

When a certain amount of DCMU, sufficient to inhibit electron transport significantly, is present in the leaf, fluorescence induction is fast in both the wild type and the triazine-resistant biotype of *Chenopodium album*. In case atrazine is used instead of DCMU, the wild type still shows the same fast fluorescence induction pattern. In the resistant biotype, however, electron transport is not inhibited. The fluorescence induction in the presence of any triazine herbicide shows basically the same pattern as the control without herbicide.

5.5 Results

5.5.1 Nucleic acid isolation

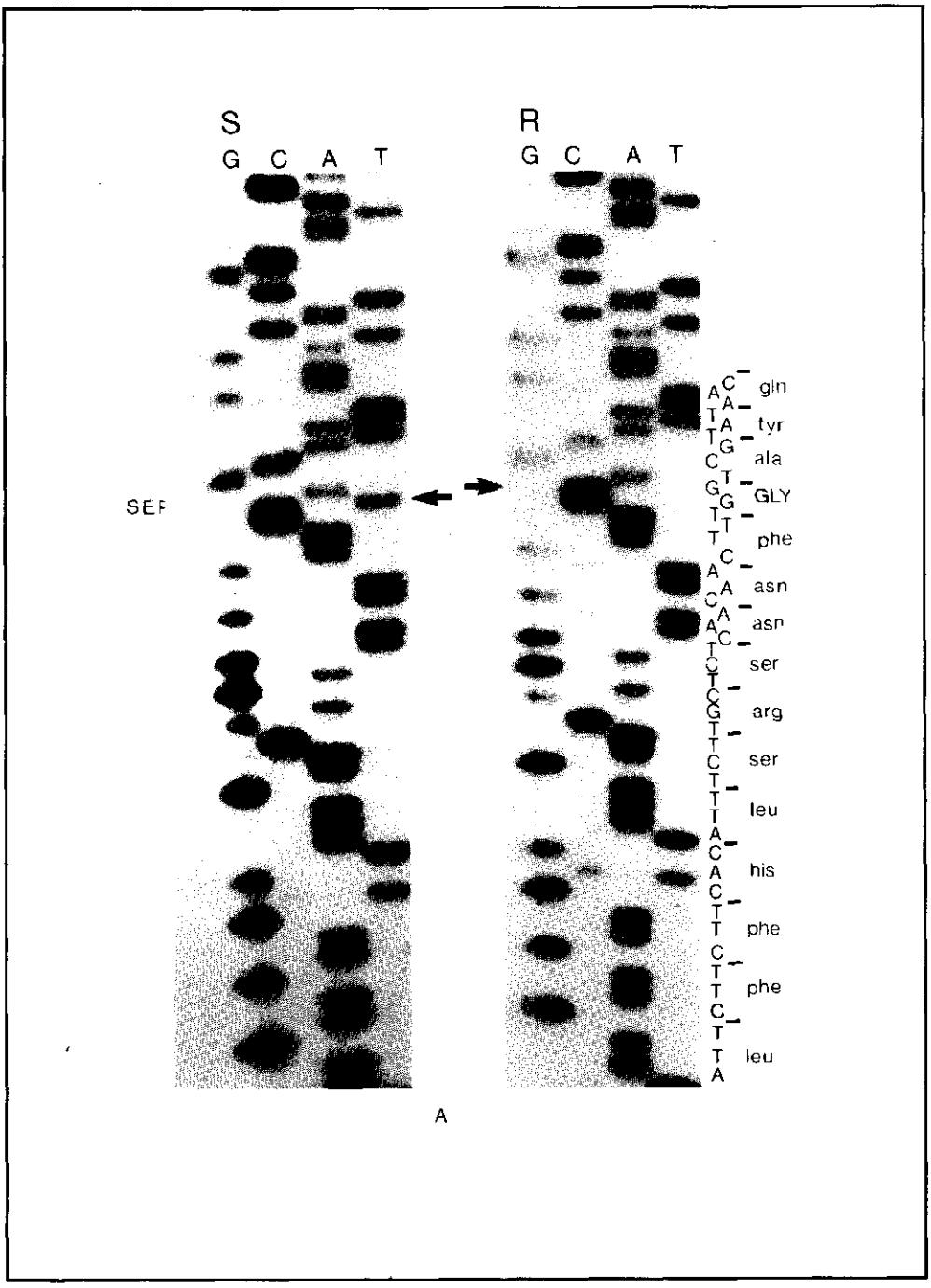
Using 10 g (wet weight) of fresh leaves 100-150 µg DNA and 3-4 mg RNA were isolated. The isolated DNA could be cut completely with restriction endonucleases BamHI and EcoRI without additional purification steps (data not shown).

In the RNA preparation several distinct bands of rRNA could be discriminated by agarose gel electrophoresis (data not shown). This indicates that no significant breakdown by RNAses has occurred.

5.5.2 Sequence analysis

Using the described primer the psbA sequence could be determined in the region coding for D1 amino acid residues 280 down to about 220. In Fig. 5.2 autoradiographs are shown from DNA and RNA sequence analyses.

Fig. 5.2A. Autoradiographs of Chenopodium album RNA. Arrows indicate the site of mutation. S=sensitive, R=resistant biotype. The letters above the lanes indicate the nucleic acids of the synthesized cDNA strand, whereas beside the figure the complementary mRNA-sequence is listed. The amino acid sequence is indicated in 3-letter code.


S
G C A T
R
G C A T
SEF
ACA gln
TAA tyr
TGC ala
CTG GLY
GGT phe
TTC asn
AAC asn
CAA ser
ACT arg
TCT ser
CGT leu
TTC his
TTA phe
ACA phe
CAC leu
CTT
TTC
TTA

A

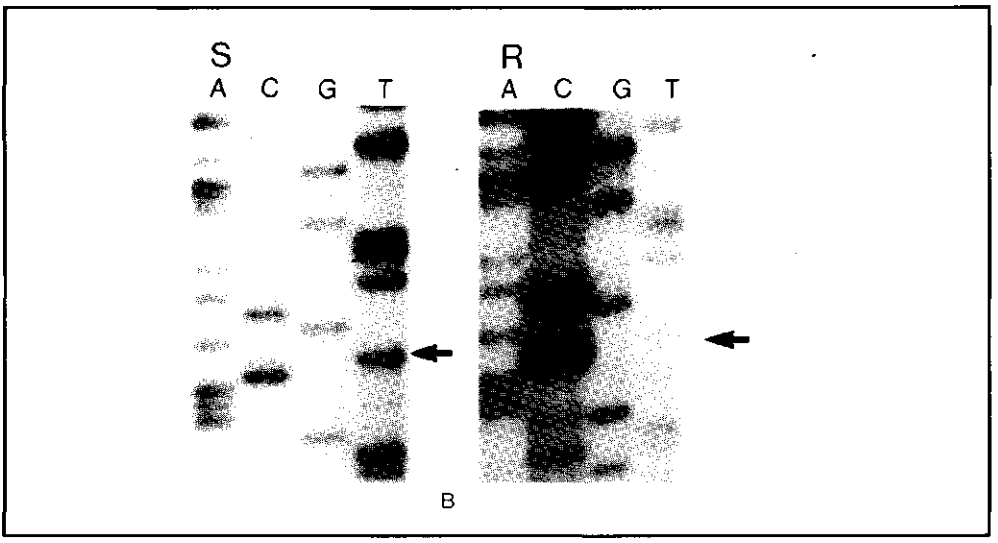


Fig. 5.2B. Autoradiograph of Chenopodium album DNA. For legend see Fig. 5.2A.

The results are summarized in Table 5.2. The sequence of *Chenopodium album* is compared to those of *Amaranthus hybridus* and *Chlamydomonas reinhardii.*

Wildtype and a triazine-resistant biotype are compared to *Chlamydomonas reinhardii*, *Amaranthus hybridus* and another line of *Chenopodium album.* Indicated are nucleotides different from those found at the corresponding positions in *Chlamydomonas reinhardii* psbA. Numbers indicate the amino acid positions (Bettini et al. 1987). The codon 264 causing a ser-gly mutation is indicated in bold lettering.

1 = *Chlamydomonas reinhardii* (Erickson et al. 1984).
2 = *Amaranthus hybridus* (Hirschberg and McIntosh 1983).
3 = *Chenopodium album* (Bettini et al. 1987).
S = *Chenopodium album*, wildtype.
R = *Chenopodium album*, triazine-resistant biotype.

Table 5.2. Partial sequence of the psbA gene of Chenopodium album.

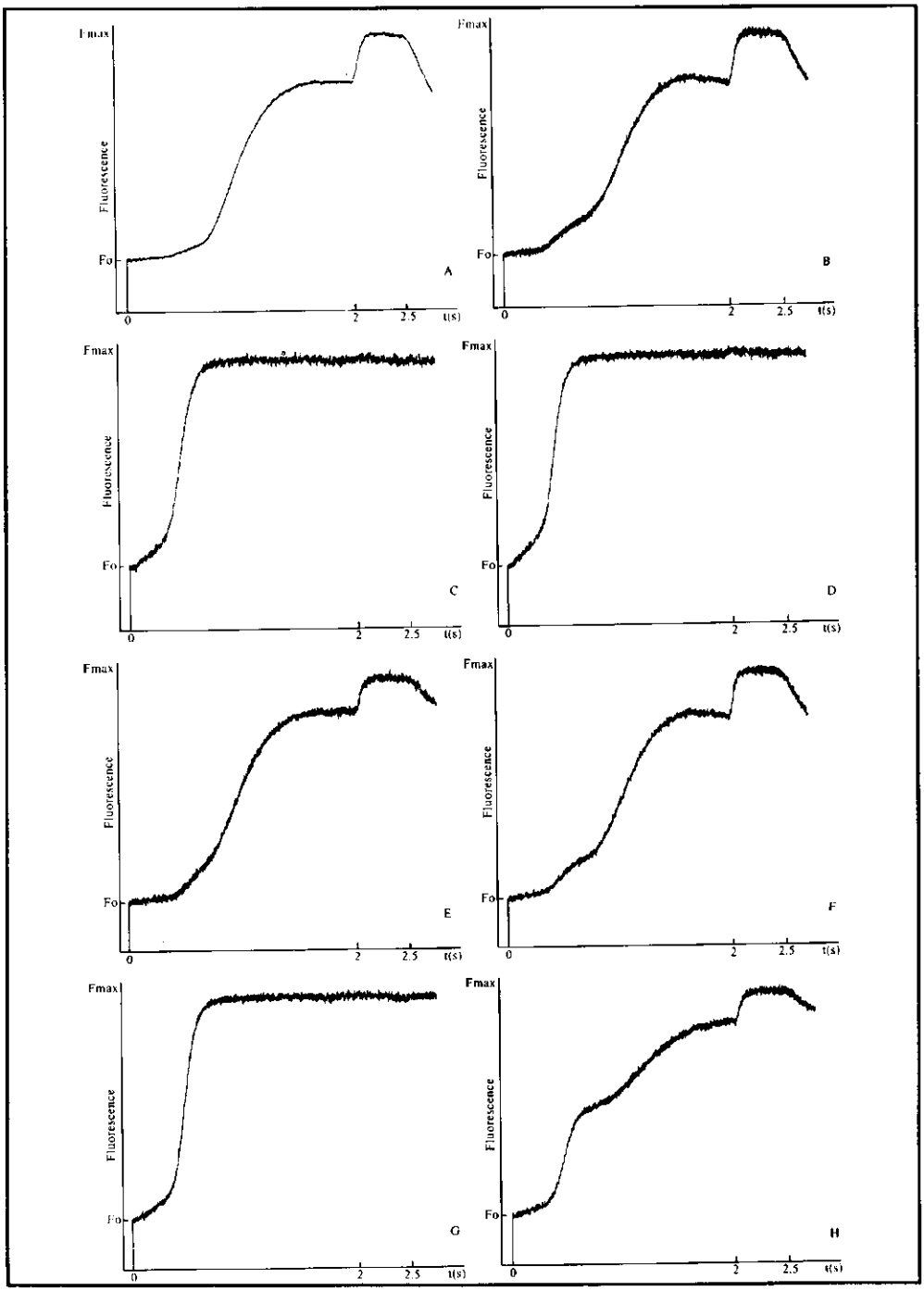
```
5'                  230                                                 240
1 GAA ACA ACT GAA AAC GAA TCA GCT AAC GAA GGT TAC CGT TTC GGT CAA GAA
2       C   A       T       T       C   A         A A       T       G
3       T   A       T       T       T   G         A A       G       G
S       T   A       T       T       T   G         A A       G       G
R       T   A       T       T       T   G         A A       G       G

                                    250
1 GAA GAA ACT TAC AAC ATT GTA GCT GCT CAT GGT TAC TTT GGT CGT CTA ATC
2               T   C   C                       T   T   T   A T G
3               T   T   T                       T   T   C   A T G
S               T   T   T                       T   C   C   A T G
R               T   T   T                       T   C   C   A T G

  260               264                         270
1 TTC CAA TAC GCT TCT TTC AAC AAC TCT CGT TCA TTA CAC TTC TTC TTA GCT
2   T       T     AGT                     T
3   T       T     AGT                     T
S   T       T     AGT                     T
R   T       T     GGT                     T
```

5.5.3 Chlorophyll *a* fluorescence induction

The chlorophyll *a* fluorescence induction shows no significant differences between the control curves of the susceptible and the resistant biotype of *Chenopodium album* (Fig. 5.3 A-B). The area over the induction curve is equal for both plants. Also the incubation of leaves in a 10 μM DCMU solution does not allow easy discrimination between both biotypes (Fig. 5.3 C-D). The sensitivity for DCMU is more or less the same for R and S, resulting in a fast rise in the induction of fluorescence in both plants. However, when leaves are incubated in a 1 μM atrazine solution a slightly faster fluorescence rise is observed for the wildtype. The faster rise as compared with the control is caused by inhibition of the reoxidation of Q_A^-. The resistant biotype displays an almost unaltered pattern even after incubation with 100 μM atrazine as compared with the control experiment. Only a small decrease of the area over the curve is observed when the atrazine concentration is increased. This indicates that almost no inhibition occurs. A further increase of the fluorescence level upon illumination with a saturating pulse means that Q_A was not yet completely reduced, i.e. inhibition was less then 100 %. The fluorescence induction curves are given in Fig. 5.3 E-H (next page).

One feature which can be observed in all curves is the small but relatively fast rise in fluorescence level after about 1 s of illumination. This rise is significant only in the curves from the resistant biotype. The cause is the shift in redox equilibrium between Q_A and Q_B, causing a more negative state of Q_A in the resistant biotype. This induces a faster rise in fluorescence as compared to the wildtype.

Fig. 5.3 A-H. Fluorescence induction curves of leaves of Chenopodium album. Curves A, C, E and G: wildtype; B, D, F, H: triazine-resistant biotype. Curves A-B: control incubation with water; C-D: 10 μM DCMU; E-F: 1 μM, and G-H: 100 μM atrazine. Actinic light was switched on at time t=0; a saturating pulse of white light (duration 0.5 s) was given at t=2 s.


Fmax
Fluorescence
Fo
A
0
2
2.5
t(s)
Fmax
Fluorescence
Fo
B
0
2
2.5
t(s)
Fmax
Fluorescence
Fo
C
0
2
2.5
t(s)
Fmax
Fluorescence
Fo
D
0
2
2.5
t(s)
Fmax
Fluorescence
Fo
E
0
2
2.5
t(s)
Fmax
Fluorescence
Fo
F
0
2
2.5
t(s)
Fmax
Fluorescence
Fo
G
0
2
2.5
t(s)
Fmax
Fluorescence
Fo
H
0
2
2.5
t(s)

5.6 Discussion

In the part of the *Chenopodium* psbA gene analyzed here, in addition to codon 264, 21 nucleotide differences were detected compared to *Chlamydomonas*. This indicates more than 85 % homology on the DNA level. Only 8 bases differ from the sequence as found in the corresponding part of *Amaranthus hybridus*, and 1 mutation distinguishes our *Chenopodium album* from another line of *Chenopodium album* described earlier (Bettini et al. 1987). These mutations are all silent, i.e. none of them leads to a different amino acid sequence of the D1 protein. In the mutant DNA the adenosine residue at position 790, the first base of the codon AGT coding for ser-264 in the wildtype, is changed to guanosine. The codon GGT which is then formed causes incorporation of glycine in the D1 protein. Apparently this changes the binding environment for the triazine herbicides, causing the observed resistance.

In the RNA gel from Fig. 5.2A it can be seen that in the sensitive biotype at position 790 two bands (A and G) are found, indicating a mixed population of mRNA molecules. No evidence was found that also on the DNA level two almost identical stretches exist. However, their existence cannot be ruled out. The double band which was seen on the RNA level could then possibly be the result of different rates of transcription of both pieces of DNA. In Chapter 6 this observation is discussed in more detail.

6 General discussion

6.1 Introduction

In this Chapter the results described before will be discussed in more detail. A new model is proposed for the electron transport at the acceptor side of PS II, in which an active role is reserved for the non-heme iron atom which is located between both quinone acceptors. Furthermore structural comparisons are made between inhibitor molecules and the secondary acceptor.

6.2 The redox state of the photosystem II acceptor complex

On the basis of measurements of flash-induced oxygen evolution patterns a reaction model was originally proposed by Kok et al. (1970). In this model the damping of the 4-step oscillation was explained by assuming fractions of the reaction centers to make either no turnover or a double turnover upon illumination with a single saturating flash. After dark adaptation, a certain fraction of the OEC's was assumed to be in state S_0 rather than in the more stable S_1, in order to get an explanation for the rather high amount of oxygen observed on the 4^{th} flash. The basis of this elegant model still seems to be valid, but with some modifications the experimental oxygen evolution patterns can be described more accurately.

This thesis provides a model which describes the competitive exchange of herbicides with plastoquinone at the PS II acceptor side (Pfister and Arntzen 1979, Vermaas 1984). In Chapter 3 an attempt has been made to characterize flash-induced oxygen evolution in the absence of inhibitors, and in Chapter 4 the data obtained were used to determine herbicide exchange rates.

A serious disadvantage of the Kok model was that no explanation was given for the fact that the flash frequency has a strong influence on the relative peak heights, at least in the range between about 10 Hz and 0.1 Hz. The slow relaxation of states S_0, S_2 and S_3 of the oxygen evolving complex to the stable S_1 cannot be held responsible for this observation. This dark adaptation process has a half-time in the range of minutes, or maybe even hours. An intrinsic electron donor D was proposed by Vermaas et al. (1984a) to account for the observed frequency-dependency of the oxygen evolution patterns. This donor was recently identified as a tyrosine residue of the D1 protein (Debus et al. 1988, Vermaas et al. 1988). Introduction of this function for D avoided the necessity to assume two different oxidation states of the OEC, S_0 and S_1, to exist in the dark.

It furthermore seemed unlikely that the probability for a miss-hit would be the same throughout a certain number of flashes. It is likely to be influenced by the redox state of the acceptor complex, which shows a binary oscillation (Velthuys and Amesz 1974). Therefore initially we used two values of α: one (α_{ox}) connected to the oxidized acceptor complex, and one (α_{red}) to the semi-reduced form. This showed an improvement in the fits obtained when experimental and theoretical oxygen evolution patterns were compared. However, the α_{red} still appeared to vary with a binary oscillation. The value found for α_{ox} was very low, and no distinct oscillation could be observed. The conclusion from these observations was that not only the acceptor side of PS II, but also the water splitting side has a distinct influence on the miss probability. This led us to the assignment of four different α-parameters. These parameters are indicated as α_n (n is 0 to 3), and are related to each of the S_n to S_{n+1} state transitions. The influence of the redox state of the OEC on the miss parameters has been suggested before (Bouges-Bocquet 1980, Delrieu 1983, Renger and Hanssum 1988). In fact the four α-parameters still describe a simplification of the actual situation. The influence of electron donation by donor D to S_2 or S_3 on the miss parameters is not included in the model. However, the assumption of only four different parameters seems justified, since donor D is active only in a small number of centers, especially when higher flash frequencies are used and D has little chance of being active. Furthermore, calculating oxygen evolution on 10

subsequent flashes does not permit to make a reliable and reproducible discrimination between 8 possible different miss-parameters. Elongation of the flash train is not of much help, since after 10 or 15 flashes hardly any oscillation is observed in the patterns.

In case of a low flash frequency, it can be assumed that already after 1 or 2 flashes all of the reduced D molecules have reduced S_2 or S_3. In the centers where this has occurred the values for the miss parameters should be shifted one step. E.g. after the first flash after dark adaptation the then oxidized acceptor (see Chapter 3) is now connected to state S_1 instead of S_2. Correspondingly, the miss parameter α_1 for this center is then low, whereas normally a high value is found for α_1. At very low flash frequencies, when the back reactions are nearly completed in the time interval between flash 1 and 2, fits should result in values for α_0 and α_2 which are about 30 % (the amount of centers containing reduced D after dark adaptation) of the respective value for α_1 and α_3. This observation was not included in the model of Chapter 3, since interpretation of data would be much more complicated compared to the extra accuracy the modification would provide.

The most striking observation made in Chapter 3 was the high value of the miss parameter α on odd flash numbers after dark adaptation. This was explained by assuming that the dark incubation conditions were favoring a relatively reduced state of the acceptor complex. The fraction of the centers with the electron located on Q_A will temporarily be unable to perform a stable charge separation, causing a high miss probability. Upon illumination centers with the electron on Q_B can make a single turnover, resulting eventually in formation of plastoquinol. This is subsequently exchanged for an oxidized PQ from the pool. The now oxidized complex has a lower miss probability, reflected in a lower value for α on the even flashes.

Another explanation for the observation of a high miss probability on odd flashes can be given by a modified model for electron transport at the PS II acceptor side. In this model a non-heme iron is included as a redox intermediate and electron carrier, as was suggested before (Jursinic and Dennenberg 1988). In the model described here the PS II acceptor complex is assumed to be in a state $Q_A.Fe^{2+}.Q_B$ after dark adaptation,

which was the situation as reported by most authors (e.g. Renger et al. 1987). The situation can be represented as an equilibrium, with an electron located either on Q_A, Q_B or the Fe-atom. The mechanism of reductant-induced oxidation, which was reported for several artificial quinones (Petrouleas and Diner 1986, Zimmermann and Rutherford 1986), may also occur with the native plastoquinone. After illumination with a single flash the primary acceptor is reduced. An electron present on Q_A is then proposed to be able to induce a reduction of Q_B by Fe^{2+}, resulting in Q_B^- and Fe^{3+}. The electron from Q_A^- should then be able to provide the second reducing equivalent to form Q_B^{2-}, thus inducing plastoquinol formation after a single flash after dark adaptation. In such a situation this mechanism possibly can account for an apparent one-step advancement of the redox state of the quinone complex in the dark. Compared to the oxidation state that is expected on account of the number of flashes fired, or the corresponding S-states of the OEC, the redox state of the Q_A.Fe.Q_B complex has been advanced once more. This alternative model is represented in Fig. 6.1.

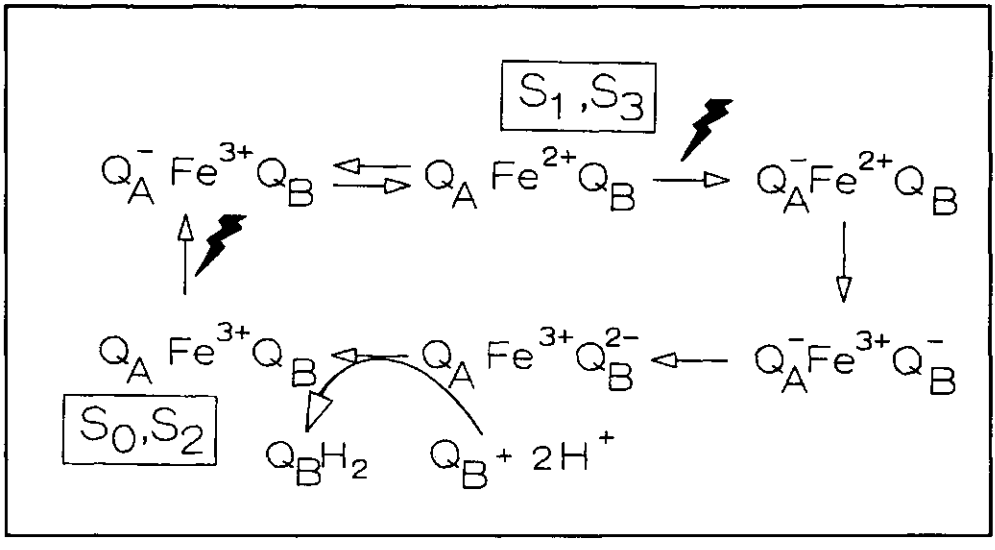


Fig. 6.1. Possible scheme for reactions at the photosystem II acceptor side in relation to the S-states of the OEC.

Thus, this model could explain why a high α_1 and α_3 (on odd flashes) are calculated, while α_0 and α_2 approach 0, as is observed in all experiments without herbicide. The value of α_n is determined mainly by the position of the equilibrium between $Q_A.Fe^{2+}.Q_B$, $Q_A^-.Fe^{3+}.Q_B$ and $Q_A.Fe^{3+}.Q_B^-$. Only the fraction with an electron on Q_A at the moment of excitation has an increased miss probability.

As outlined above, a high value of α may be due to a high fraction of centers with an electron on Q_A. Another possible explanation is that the charge of the Fe-atom causes an equilibrium shift in the redox state of the Q_A-pheophytin complex. An oxidized Fe^{3+} as found after odd flashes (according to Fig. 6.1) could shift the equilibrium towards a more oxidized state on the pheophytin, resulting in a decrease of the miss probability on even flashes. The α_3 parameter is found to be a little higher than α_1, which may be caused by the fact that the S_3 to S_0 transition is about 10-fold slower than the S_1 to S_2 transition (Renger 1988).

The possibility that this mechanism occurs only in a fraction of the reaction centers cannot be ruled out. Several authors have provided evidence that a non-heme iron atom is found in only about half of the acceptor complexes (Jursinic and Dennenberg 1988, Aasa et al. 1989).

6.3 The influence of herbicides on reactions at the acceptor side

In Chapter 3, a model is described which explains the patterns of flash-induced oxygen evolution in the absence of inhibitors (indicated with I). The values of the miss parameters on odd flashes after dark adaptation (α_1 and α_3) were then found to be high. However, in the presence of a herbicide dark incubation seemed to result in lower values for these parameters (Chapter 4). This finding can be explained by assuming that incubation with a herbicide, even at low concentrations, can result in a shift in the redox state at the PS II acceptor complex towards a more oxidized state. A more oxidized state of the acceptor complex could be explained using the classical concept of electron flow through Q_A and Q_B (i), as well as by including a function of the non-heme iron in the

model of electron flow between Q_A and Q_B (ii), which was discussed in paragraph 6.1.

(i). In Chapter 3 a semi-reduced quinone complex $(Q_A.Q_B)^-$ was proposed to exist in the dark. Incubation with a herbicide induces an electron shift towards Q_A, as was shown before (Velthuys and Amesz 1974, Jursinic and Stemler 1983). This situation is not stable in the dark, which is reflected by the fact that an oscillation in herbicide binding and in fluorescence intensity could only be observed for a short time (in the second time range) after herbicide addition (Velthuys and Amesz 1974, Laasch et al. 1983, Jursinic and Dennenberg 1988). Measurement of chlorophyll *a* fluorescence in thylakoids, incubated with DCMU and preilluminated with one single flash, also showed that in the dark the negative charge on Q_A^- was dissipated by donation to an unknown endogenous electron acceptor with a half-time of several seconds (Robinson and Crofts 1983, Ooms, pers. comm.). This can be represented by the following reaction sequence:

$$\langle Q_A.Q_B\rangle^- \xrightarrow[Q_B]{I} Q_A^-.I \xrightarrow{e} Q_A.I \qquad (6.1)$$

In the state Q_A.I, the inhibitor may exchange for Q_B, resulting in the state $Q_A.Q_B$. However, because of the long residence times of the herbicides used (see Paragraph 6.4), most acceptor complexes will be in the state Q_A.I. Dark reduction of $(Q_A.Q_B)$ as was assumed to occur slowly in the absence of herbicides (Chapter 3), probably has a half-time of more than 30 s. Thus after dark incubation in the presence of an inhibitor most acceptor complexes may be assumed to be in the state $Q_A.Q_B$ or Q_A.I, resulting in a low value of the miss parameter on the first flash.

After one flash after dark adaptation the centers containing an inhibitor will have a value for the miss parameter of (almost) 1, due to formation of Q_A^- which cannot be reoxidized. This has no influence on the determination of the parameters, since oxygen evolution is measured and the inhibited centers cannot be monitored. Thus only the amplitude, but not the relative peak height in an oxygen evolution pattern is altered.

(ii). When the mechanism of reductant-induced oxidation, as described in paragraph 6.1, is assumed to occur, the observation that incubation with herbicide causes formation of a more oxidized acceptor complex in the dark can be explained as well. Starting in the dark adapted state $Q_A.Fe^{2+}.Q_B$, addition of a herbicide (I) leads to an exchange of Q_B for I (Eqn. 6.2). Displacement of the plastoquinone by a herbicide could result in destabilization of the acceptor complex $Q_A.Fe^{2+}$, which is now deprived of the electron-stabilizing Q_B. If the Q_A midpoint potential is lowered by addition of a herbicide, as was proposed by Velthuys and Amesz (1974), this may result in a herbicide-induced reduction of Q_A by the iron atom. Since Q_A^- is not stable for longer than several seconds, the result might be a slow oxidation in the dark of the Q_A.Fe acceptor complex. This is represented in Eqn. 6.2:

$$Q_A.Fe^{2+}.Q_B \overset{I}{\underset{Q_B}{\rightleftharpoons}} Q_A.Fe^{2+}.I \longrightarrow Q_A^-.Fe^{3+}.I \overset{}{\underset{e}{\rightleftharpoons}} Q_A.Fe^{3+}.I \qquad (6.2)$$

Incubation with a herbicide could thus possibly induce oxidation of Fe^{2+} to Fe^{3+}. If subsequently the herbicide is exchanged for Q_B, the resulting complex is the fully oxidized state $Q_A.Fe^{3+}.Q_B$. The final result of herbicide addition in the dark is an oxidation of the acceptor complex leading to $Q_A.Fe^{3+}.I$ or $Q_A.Fe^{3+}.Q_B$. These states are related to a low value of the miss probability on the first and subsequent odd flashes after dark adaptation.

It is worthwhile to try to explain the results of Velthuys and Amesz (1974) in the light of a possible action of the non-heme iron in electron flow between Q_A and Q_B. These authors observed an increase of chlorophyll fluorescence induced by addition of dithionite in darkness. The fluorescence increase induced by dithionite added after preillumination with short flashes was dependent on the flash number with a periodicity of two; it was large after an odd number of flashes, and small after a long dark-time or after an even number of flashes. The same results were obtained by addition of DCMU instead of dithionite.

As was discussed in Paragraph 6.1 (Fig. 6.1), after long dark adaptation and also after an even number of flashes the acceptor complex is in the state $Q_A.Fe^{2+}.Q_B$, which is in equilibrium with $Q_A^-.Fe^{3+}.Q_B$.

Addition of dithionite to this state should lead to $Q_A^-.Fe^{2+}.Q_B$, which is related to a (probably small) increase in fluorescence. Fig 6.1 shows that after an odd number of flashes the acceptor complex is in the state $Q_A.Fe^{3+}.Q_B$, which is more oxidized than the state after darkness or after an even number of flashes. Consequently, addition of dithionite to the state $Q_A.Fe^{3+}.Q_B$ leads to more reduction of this complex, or rather of Q_A. This results in a larger increase of fluorescence after an odd number of flashes compared to that after darkness or an even number of flashes.

The comparable results obtained by adding herbicides instead of dithionite (Velthuys and Amesz 1974, Jursinic and Dennenberg 1988) were explained by these authors by assuming that the midpoint potential of Q_A is lowered by these herbicides. The model of reductant-induced oxidation (Fig. 6.1) can be used to explain these phenomena only if a reduction of the acceptor complex upon herbicide addition is assumed to occur, analogous to the reduction by dithionite. Such a reduction of the acceptor complex by a herbicide may be caused by a herbicide-induced shift in the redox potential of the Q_A-environment. A possible electron donor might be the reaction center itself (maybe via the primary donor or the secondary donor D), or any side group of a neighboring amino acid residue.

6.4 Structure comparisons between herbicides and plastoquinone

The herbicides used in this study all displace the PS II secondary quinone acceptor, by either occupying or modulating its binding niche. Recently, Gardner (1989) proposed a model accounting for binding of various compounds to slightly different, overlapping regions of the plastoquinone site. In addition to the herbicides mentioned in this study, also ioxynil and several cyanoacrylate herbicides match (part of) the plastoquinone structure, as shown in Fig. 6.2. In the Appendix molecular fitting models are shown of the structural resemblances between Q_B and the herbicides used in this study. The hypothesis that all PS II inhibitors bind to different parts of the D1 protein (for a review see Van Rensen 1989) should be reconsidered critically in view of recent research. The

observations on which the model of allosteric interaction was based are not quite convincing.

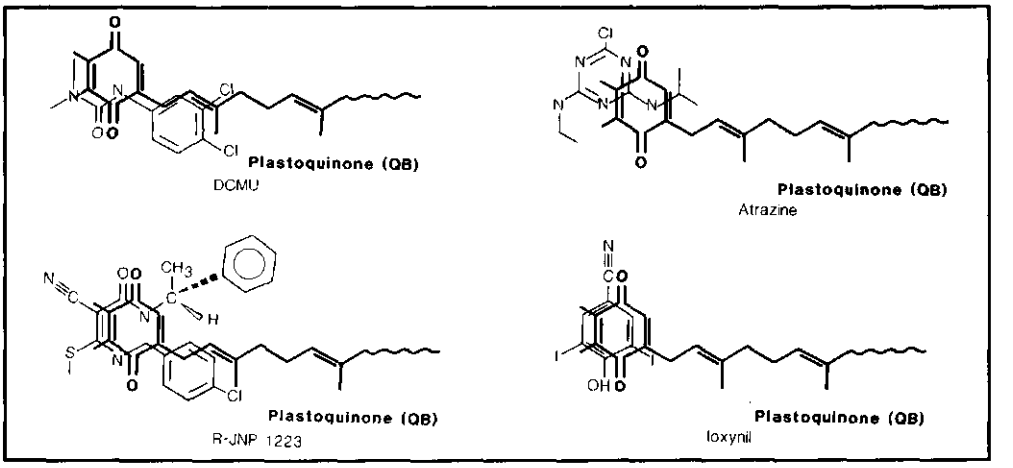


Fig. 6.2. Conformation of several PS II inhibitors as compared to that of plastoquinone. See Appendix for computer simulations.

The widely accepted view of competitivity of different chemical classes of inhibitor compounds with each other (Tischer and Strotmann 1977, Laasch et al. 1982), with plastoquinone electron acceptor analogues (Oettmeier and Soll 1983, Vermaas et al. 1983, Pfister and Schreiber 1984), or with formate and bicarbonate (Govindjee and Van Rensen 1978, Van Rensen and Vermaas 1981, Snel and Van Rensen 1984) demonstrates a very close interaction of these compounds. They were all proved to bind in some way specifically to the D1 protein. For all groups of compounds different binding niches were supposed to influence each other by allosteric modification of neighboring sites. According to the model of Trebst (1987) all herbicide and quinone sites are found to map in the region between helices 4 and 5 of the D1 protein. It seems unlikely, though, that this relatively short chain of about 60 amino acids can be held to form so many different high-affinity niches, and to account for such intricate allosteric interactions. Therefore a common binding environment for (almost) all herbicides and quinones may be supposed to

exist, with the possible exception of a separate binding niche for phenolic herbicides between the D1 and D2 proteins (Trebst 1987). Bicarbonate and formate seem to compete for a third binding site, which is possibly related to the non-heme iron between Q_A and Q_B (Van Rensen et al. 1988, Nugent et al. 1988).

Azido-labeled DCMU-analogues were proposed to occupy at least two different binding sites located on different PS II reaction center proteins (Boschetti et al. 1985). Azido-labeled compounds can bind almost all groups neighboring the azido group, and care must be taken with the interpretation of the results (Pfister et al. 1981, Gardner 1981, Gressel 1982, Oettmeier et al. 1984). The observations of Boschetti et al. (1985) may be explained by assuming binding of the azido-labeled compound at a site other than that at which electron transport inhibition is caused. Different binding sites, with correspondingly different affinities, were also observed for triazine-type herbicide analogue (Oettmeier et al. 1984), phenolic compounds (Oettmeier et al. 1986) and different other inhibitor compounds (Laasch et al. 1982).

Displacement studies revealed that a phenolic inhibitor (ioxynil), an urea compound (DCMU) and a triazine (atrazine) competitively inhibit PS II electron transport (Naber and Ivens, unpublished data). Comparison of mutual displacement between these herbicides indicated no dependency of the order in which they were applied. The data suggested a common site for ioxynil, DCMU and atrazine binding in both atrazine-sensitive and -resistant chloroplasts from *Chenopodium album*. Resistance to atrazine is not caused by absence of the binding site, but rather by a very fast release from it (see Table 6.1).

The observation that plastoquinone can transfer its electrons to the cyt b_6/f complex, whereas the PS II herbicides do not seem to bind to this complex, does not prove that the binding sites at the D1 protein must be different. The amino acid sequence of both proteins (the D1 protein and the plastoquinone-binding region of the cytochrome b_6/f complex) reveals that both sites at which the electron carrier can bind must show structural differences. Interaction of the herbicides with one of these sites does not necessarily indicate a similar (high-affinity) interaction with the other.

6.5 Comparison of herbicide binding in wildtype and triazine-resistant *Chenopodium album*

The altered herbicide binding properties of triazine-sensitive and -resistant biotypes of different plants and algae are not limited to the triazine-herbicide binding or release kinetics. The serine to glycine change in the D1 protein also affects the effectiveness of other types of compounds, which can be expressed in either lower (urea, uracils, biscarbamates) or higher (urea, phenols) pI_{50}-values (Chapter 4, Oettmeier et al. 1982, Pucheu et al. 1984, Jansen et al. 1986, Van Oorschot and Van Leeuwen 1987, Solymosi and Lehoczky 1989, Erickson et al. 1989). The level of resistance to the different compounds is strongly dependent on the mutation causing it. Also the electron transport rate from primary to secondary quinone acceptor is often lower in triazine-resistant plants (Bowes et al. 1980, Jansen et al. 1986, Haworth and Steinback 1987, Johanningmeier et al. 1987, Jursinic and Pearcy 1988, Erickson et al. 1989).

The mutation in the D1 protein seems to be responsible for a change in binding and release kinetics not only of atrazine, but also of DCMU, ioxynil and the cyanoacrylates used. From Table 4.1, the average residence time of a herbicide molecule at its binding site can be calculated. These values equal the inverse of the dissociation rates $E_3 + E_4$.

Table 6.1. Values for the residence time of herbicides at the Q_B-site.

	Chenopodium S	*Chenopodium* R	R/S ratio	Resistance
Atrazine	6.7	0.058	0.0087	290
DCMU	90	500	5.6	0.16
Ioxynil	0.25	4	16.7	0.1
JNP1166	16	45	2.8	1.8
JNP1174	1.3	6.7	5.3	1.4
JNP1222	6.7	0.33	0.05	4.6
JNP1223	1	10.6	0.094	0.5

Residence times (s) in S and R chloroplasts, defined as $1/(E_3+E_4)$. R over S ratio represents the relative residence time on the mutant D1 protein as compared to the wildtype. Resistance is the factor with which the herbicide resistance is increased in the triazine-resistant chloroplasts as compared to the wildtype; see text for formula.

The value of the resistance factor is defined as the quotient of the R over S ratios of (E_3+E_4) and (E_1+E_2), i.e. the change in herbicide release- divided by the change in binding-parameters. This can be expressed as in Eqn. 6.3:

$$\text{Resistance} = ((E_3+E_4)_R/(E_3+E_4)_S)/((E_1+E_2)_R/(E_1+E_2)_S) \qquad (6.3)$$

The values which are smaller than 1 indicate an increased sensitivity in the resistant biotype. This corresponds to a higher pI_{50}-value in Table 4.2. Of course, the most striking difference is found for atrazine. The resistance factor calculated is about 300. For ioxynil, and to a lesser extent for DCMU, the wildtype is less sensitive than the mutant biotype. This phenomenon is discussed in Paragraph 6.5. From the cyanoacrylates used, the isomers JNP-1166 and -1174 show similar behavior: a slight increase in resistance in the triazine-resistant plants. However, JNP-1222 displays a 5-fold decrease in activity in the resistant chloroplasts, whereas here the isomer JNP-1223 is a stronger inhibitor.

6.6 Binding properties of atrazine, DCMU and ioxynil

As is often observed in fields where atrazine is used for a number of years in succession, the effectivity in the control of several weed species decreases dramatically. This is due to a strongly increased rate of dissociation from the binding site in plants which have developed a resistance. The association of the inhibitor seems to be influenced only marginally. An obvious explanation may be to assume that association is influenced mainly by properties of the herbicide molecule, whereas

dissociation is related to the opportunity to form stabilizing interactions with the niche. Atrazine can probably form a hydrogen bond to the hydroxyl group of the serine-264 residue in the wildtype plants, and is deprived of this possibility in the resistant biotype. This is in agreement with the suggestion of Michel (Michel et al. 1985, Sinning and Michel 1987), but provides no explanation for the recent discovery of triazine- and/or DCMU-resistant biotypes where serine is replaced with threonine at the 264-position of the D1 protein of tobacco (Shigematsu et al. 1989) and *Euglena* (Johanningmeier, pers. comm.). The resistance of the threonine-mutants is perhaps due to a slight conformational change, which places the hydroxyl group in a less favorable position to interact with triazines.

The observation that the wildtype *Chenopodium* is less sensitive towards DCMU is not in agreement with earlier oxygen production measurements (Pfister and Arntzen 1979, Jansen et al. 1986). This discrepancy can possibly be explained by a difference in measuring conditions. Most authors used continuous illumination, whereas in our experiments flash-induced oxygen evolution was monitored. Under circumstances of continuous illumination the relative importance of E_4 is higher, but E_3 is more important in flash-induced measurements. The change in inhibitory properties between resistant and sensitive biotype can thus be ascribed to a decrease of the herbicide dissociation of an oxidized acceptor complex: the E_3-parameter is reduced in the resistant biotype, resulting in stronger inhibition (see Table 4.1). For ioxynil the decrease in I_{50}-value in the resistant biotype was also measured under continuous illumination, but to a lesser extent (Jansen et al. 1986). The apparent increase in sensitivity of the resistant biotype for ioxynil under our measuring conditions can be explained in a similar way as described above for DCMU.

6.7 Binding properties of the cyanoacrylate compounds

Properties and structure-activity relationships of many cyanoacrylate herbicides are described in detail in a number of papers (Phillips and

Huppatz 1984, 1987, Huppatz and Phillips 1984, 1987a, b, c). Isomers like JNP-1166 and JNP-1174 were shown to be able to show a different binding behavior between the D1 protein from an atrazine sensitive and a resistant biotype of *Brassica napus* (Phillips and Huppatz 1987). The differences between S and R stereo-isomers seemed to be less pronounced in the resistant biotype of *Chenopodium album* than in the wildtype or in pea. This indicates that a group attached to the chiral carbon atom may interact with the ser-264 residue of the D1 protein. This seems unlikely for the alkyl substituents commonly found in this position. The effect of a serine to glycine mutation could be a decreased influence of the conformation around the chiral carbon atom, while the overall binding affinity stays more or less equal. A conformational change in the D1 protein could also account for the various levels of cross resistances found in different algal and higher plant triazine-resistant biotypes (Oettmeier et al. 1982, Pucheu et al. 1984, Jansen et al. 1986, Solymosi and Lehoczki 1989).

Cyanoacrylates with an ethoxyethyl ester group, like JNP-1166 and JNP-1174, are among the most powerful PS II inhibitors. The ester group appears to have a strong influence on the pI_{50}-value of the herbicide. From comparison of possible binding orientations it seems as if the ester side chain of cyanoacrylates may interact with residue 264. The chiral C-atom would then be positioned at a relatively short distance from this ester, thus explaining the differences in relative activities of S and R isomers in sensitive and resistant plant biotypes.

Isomers JNP-1222 and JNP-1223 are lacking the ester group. The effectivity of the compounds is decreased significantly in all plants tested. The distinction in activity between both isomers is less pronounced. However, the relative resistance of the *Chenopodium* wildtype and the triazine-resistant biotype is more significant than within the other pair of isomers. The dissociation rate of JNP-1222 is much enhanced in the resistant chloroplasts, and can be held responsible for the decrease in herbicidal properties. This may be the result of less specific interaction with distinct amino acid residues, increasing the influence of the molecular shape on the binding characteristics.

6.8 Sequence analysis of wildtype and mutant DNA and RNA

The analysis of the RNA from the wildtype led to an interesting observation. Next to the band on the gel indicating the adenosine residue in position 790, at the same position guanosine (as present in the mutant RNA) was found. Intensities of both bands were more or less equal (Fig. 5.2). This means that a mixed population of RNA molecules is present. However, the DNA of the same plants showed no such ambiguity. Here only the expected adenosine residue was found in the wildtype, and guanosine in the resistant biotype.

The presence of two different types of RNA to which our probe hybridizes in the wildtype is rather puzzling. Assuming that both bands originate from intact copies of the psbA gene, it indicates that in the wildtype plants the mutant RNA is not translated into a functionally active protein, since triazine-resistance would then be observed. This is not the case: the difference in resistance level between both biotypes is about 1000-fold (Jansen et al. 1986). The most obvious explanation would be the assumption that a mixed population of S and R plants has been used, or that leaves were mixed up. This can easily be ruled out, because in that case the same phenomenon would have been observed in the simultaneously isolated DNA. Accidental mixing up of isolated nucleic acid preparations must also be ruled out, since the intensity of the extra band is much stronger than could be expected from a contamination.

A gene heterogeneity can be ruled out for the same reason as mixing up of plants: no aberration is found in the DNA analysis. It might be argued, though, that different transcription rates are causing the appearance of the double band on the RNA gels. Occurrence of two (identical) copies of the psbA gene is normal in algae, where the gene is located in the inverted repeat. In higher plants, this was never observed. Furthermore it seems unlikely that the genetic mutation occurs in the wildtype, without being expressed as resistance.

A related phenomenon was recently observed by Bettini et al. (1988). They discovered that a piece of ctDNA is present in the mitochondrial DNA of *Chenopodium album*. The fragment is homologous to the 3'-end of the psbA gene. It is expressed as a part of a 0.8 kb transcript, but only

in the triazine-resistant biotype and not in the wildtype. The transcript is found to be present only in the mitochondria. No function has as yet been ascribed to the RNA, and no direct involvement in triazine-resistance could be demonstrated.

However, the observations could be an explanation for the relatively easy development of resistance as a result of spraying repeatedly with triazine herbicides. Also it might provide an explanation for the fact that in almost all higher plants sequenced so far the same mutation was found to cause triazine resistance. Further experiments are needed to clarify the observed phenomenon.

Summary

Many weed-controlling agents act by inhibiting the process of photosynthesis. Their mode of action is a displacement of the secondary quinone electron acceptor of photosystem II from its proteinaceous binding environment. This results in a blocking of the electron transport. Consequently plants are no longer able to produce ATP and to reduce $NADP^+$, which eventually leads to starvation and death.

In this thesis an attempt is made to characterize the interactions of herbicides with their binding environment. Several herbicides were used to measure binding kinetics in a wildtype and a triazine-resistant biotype of Common Lambsquarters, *Chenopodium album* L.

In Chapter 1 a general introduction is given on photosynthesis, chloroplast structure and function, the chloroplast genome, and herbicide action and resistances.

Chapter 2 describes the methods used to isolate chloroplasts and to measure oxygen production. Also the fluorescence induction measurements are described, which can be used as a rapid method to discriminate between herbicide-sensitive and -resistant plants. Procedures for the simultaneous isolation of total plant nucleic acids, for the separation of DNA and RNA and for the respective sequence analyses are outlined.

In Chapter 3 a model is described which can be used to simulate flash-induced oxygen production by isolated thylakoids. In the literature a model was described before, which explains the observed 4-step oscillation in flash-induced oxygen evolution by assuming the existence of 4 different S-states of the oxygen evolving complex. This model is refined here by assuming the values of the miss parameters α to be dependent on both the redox state of the quinone acceptor complex (Q_A.Fe.Q_B) of photosystem II and on the S-state transition involved. The best fit between theoretical and experimental oxygen evolution patterns is obtained when 4 different miss parameters are distinguished, corresponding with the 4

S-state transitions of the oxygen evolving complex. The values of two of these α parameters, notably those for the 2^{nd} and 4^{th} flash, are found to approach 0, while the other two have values of about 0.2 and 0.4. The value for the double hit parameter β is found to be about 0.06. The oxygen evolving complexes of thoroughly dark adapted chloroplasts are found to be for almost 100 % in the most stable single oxidized state S_1. A fraction of 30 % of the reaction centers is assumed to be connected to a one-electron donor D, which is able to reduce the S_2- or S_3-state of the oxygen evolving complex with a half time of 2-3 seconds.

In Chapter 4 the derived model is used to determine the exchange parameters of herbicides with the secondary acceptor Q_B. These exchanges, which influence the oxygen evolution patterns, can be determined by comparing experimental and theoretical patterns for various herbicide concentrations and flash frequencies. The I_{50}-values derived from the measurements are in agreement with values measured with other methods. The resistance to triazine compounds proved to be caused by an increase in the herbicide release rather than by a lower binding rate. Configuration around a chiral carbon atom, present in two pairs of isomers of cyanoacrylate inhibitors, has a strong influence on inhibition properties. The differences are largely due to alterations in the release kinetics. This observation suggests that herbicide binding is determined mainly by physical properties, like e.g. hydrophobicity. A stationary binding, resulting in a significant electron transport inhibition, requires a strict molecular shape.

In Chapter 5 partial sequence analyses from DNA and mRNA isolated from a *Chenopodium album* wildtype and a triazine-resistant biotype are presented. The only difference found was an adenine to guanine point mutation resulting in a serine to glycine alteration at position 264 in the D1 (herbicide binding) protein.

The thesis is concluded with Chapter 6, the general discussion, in which an alternative explanation for redox reactions at the photosystem II acceptor side is presented. In this model the non-heme iron, located between the primary and the secondary acceptor of photosystem II, is proposed to play an active role in *in vivo* electron transport.

Samenvatting

De werking van veel onkruidbestrijdingsmiddelen is gebaseerd op het remmen van de fotosynthese. Hun aktiviteit berust op het verdringen van de sekundaire elektronen acceptor van fotosysteem II van de eiwit-omgeving waar deze normalerwijze gebonden is. Dit resulteert in blokkeren van het elektronen transport. De plant is nu niet langer in staat om ATP te produceren en $NADP^+$ te reduceren, wat uiteindelijk het afsterven ervan tot gevolg heeft.

In dit proefschrift is getracht om de wisselwerkingen tussen herbiciden en hun bindingsomgeving te beschrijven. Verschillende typen herbiciden zijn gebruikt voor het meten van bindingskinetiek in een wildtype en een triazine-resistent biotype van Melganzevoet, *Chenopodium album* L.

In Hoofdstuk 1 wordt een algemene inleiding gegeven over fotosynthese, struktuur en funktie van de chloroplasten, het erfelijk materiaal in de chloroplasten, en de werking van en resistentie tegen herbiciden.

In Hoofdstuk 2 worden de methoden beschreven die gebruikt zijn voor het isoleren van chloroplasten en het meten van hun zuurstofproduktie. Verder is de methode voor het meten van fluorescentie-induktiekurves uiteengezet. Met behulp van deze techniek kan snel een kwalitatief onderscheid gemaakt worden tussen herbicide-gevoelige en -resistente planten. Tenslotte wordt een methode gegeven voor het gelijktijdig isoleren van DNA en RNA uit intakte planten, en voor de sequentie-analyses hiervan.

In Hoofdstuk 3 wordt een model geformuleerd voor de simulatie van flits-geïnduceerde zuurstofproduktie door geïsoleerde thylakoiden. De oscillatie met periode 4 die hierin wordt waargenomen werd door anderen verklaard door aanname van 4 verschillende S-toestanden voor het zuurstof producerend complex van fotosysteem II. Dit model wordt hier

verfijnd door aan te nemen dat de waarde van de mis-parameters α afhankelijk is van zowel de redoxtoestand van het chinon-acceptor complex (Q_A.Fe.Q_B) van fotosysteem II als van de S-toestands overgang die door de flits wordt bewerkstelligd. De beste overeenkomst tussen berekende en gemeten zuurstofproduktie-patronen wordt verkregen door 4 verschillende mis-parameters te onderscheiden, gerelateerd aan de 4 S-toestands overgangen van het zuurstof producerend complex. De waarden van twee van deze α parameters, te weten die voor de 2^e en voor de 4^{de} flits, zijn gelijk aan 0, terwijl de beide anderen respektievelijk 0.2 en 0.4 zijn. De waarde van de "double-hit" parameter β, die aangeeft in welke fraktie van de reaktiecentra op één flits 2 toestandsovergangen plaatsvinden, heeft een waarde van 0.06. De zuurstof producerende complexen van langdurig donker geadapteerde chloroplasten bevinden zich voor (naganoeg) 100 % in de meest stabiele, eenmalig geoxideerde, grondtoestand S_1. Gevonden werd verder dat een fraktie van 30 % van de reaktiecentra verbonden is met een elektron-donor D, die in staat is de S_2- of S_3-toestand éénmaal te reduceren met een halfwaardetijd van 2 tot 3 seconden.

In Hoofdstuk 4 wordt het afgeleide model gebruikt voor het berekenen van de uitwisselingsparameters van herbiciden met de secundaire acceptor Q_B. Deze uitwisselingen, die de zuurstofproduktie patronen beïnvloeden, kunnen bepaald worden door vergelijking van gemeten en berekende patronen voor verschillende herbicide-concentraties en flitsfrequenties. De I_{50}-waarden die uit deze berekeningen bepaald kunnen worden komen overeen met waarden gemeten met andere methoden. De resistentie tegen triazine-verbindingen bleek voornamelijk veroorzaakt te worden door een toename in de dissociatiesnelheid van het herbicide molecuul, en niet of nauwelijks door een veranderde associatie. De rangschikking van de verschillende groepen rond een chiraal koolstof atoom, wat in twee paren van hier gebruikte cyano-acrylaat herbiciden vóórkomt, beïnvloedt de eigenschappen als remstof sterk. De verschillen worden ook hier vooral veroorzaakt door gewijzigde dissociatie-eigenschappen. Deze waarnemingen suggereren dat de associatie van een herbicide met de Q_B-bindingsplaats vooral wordt bepaald door fysische eigenschappen, zoals bijvoorbeeld hydrofobiciteit. Voor een stabiele

binding, resulterend in duidelijke remming van het elektronentransport, is een strak bepaalde vorm van de moleculen vereist.

In Hoofdstuk 5 worden de resultaten van gedeeltelijke sequentie-analyses van DNA en mRNA, geïsoleerd uit een *Chenopodium album* wildtype en een triazine-resistent biotype, gepresenteerd. Het enige verschil wat gevonden werd was een puntmutatie van adenine naar guanine, resulterend in een verandering van serine naar glycine op positie 264 van het D1 (herbicide bindend) eiwit.

Het proefschrift wordt besloten met Hoofdstuk 6, waarin een alternatief schema voor redox reakties aan de acceptor kant van fotosysteem II gepresenteerd wordt. In dit model wordt voorgesteld dat het ijzer-atoom, dat gesitueerd is tussen de primaire en secundaire elektronen acceptor van fotosysteem II, een aktieve rol zou kunnen spelen in het *in vivo* elektronentransport.

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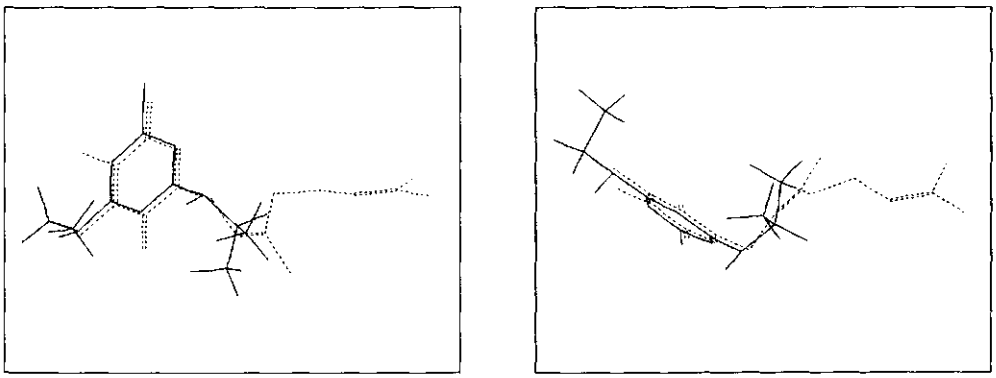
Curriculum vitae

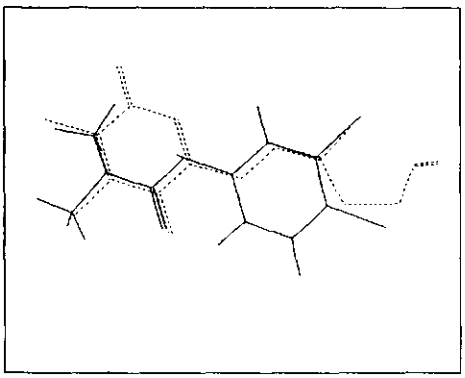
Ik ben geboren op 20 mei 1959 in Rotterdam. Het diploma Atheneum B heb ik in 1977 behaald aan het Mgr. Zwijsen College te Veghel. Daarna begon ik in Wageningen aan de Landbouwuniversiteit met de studie Moleculaire Wetenschappen. Tijdens de doctoraalstudie heb ik een 9-maands onderzoek Moleculaire Genetica uitgevoerd bij Dr. Henk van den Broek. Vervolgens kwam ik voor een 6-maands bijvak terecht op de vakgroep Plantenfysiologisch Onderzoek, waar Dr. Jan Snel mij liet kennismaken met fotosynthese. Als laatste deel van de studie heb ik 6 maanden stage gelopen bij Dr. Jan Maat van de Recombinant DNA groep van het Unilever Research Laboratiorium in Vlaardingen. In 1985 behaalde ik het Ingenieursdiploma. Daarna ben ik van 1 januari 1986 t/m 31 december 1988 als onderzoeksassistent verbonden geweest aan de vakgroep Plantenfysiologisch Onderzoek van de Landbouwuniversiteit Wageningen, waar onder leiding van Dr. Van Rensen en Prof. Vredenberg het in deze dissertatie beschreven onderzoek werd uitgevoerd. Sinds 1 mei 1989 ben ik verbonden aan de Lehrstuhl Biochemie der Pflanzen van de Ruhr-Universität Bochum (W. Duitsland).

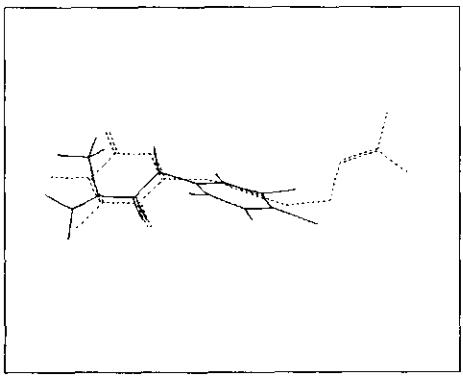
Appendix

Simulations of the structural resemblances between several photosystem II inhibitors and the intrinsic electron acceptor Q_B are presented. Solid lines represent the inhibitor molecules, dotted lines the plastoquinone. The right-hand figures are rotated about 90 degrees around the horizontal axis in comparison to the left-hand.



Atrazine.





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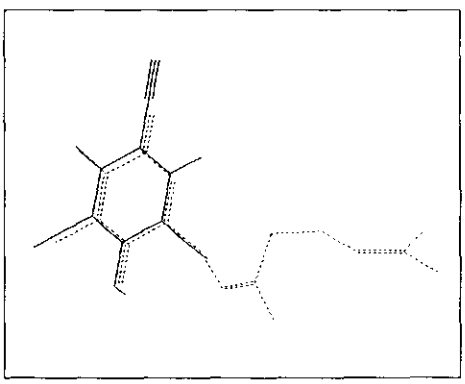
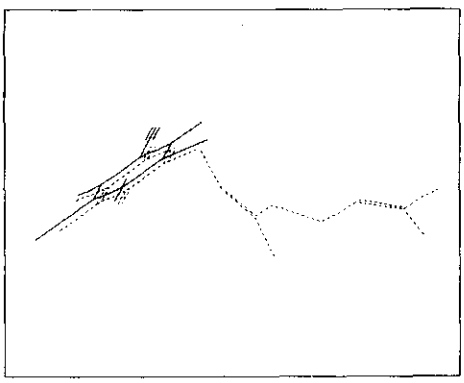
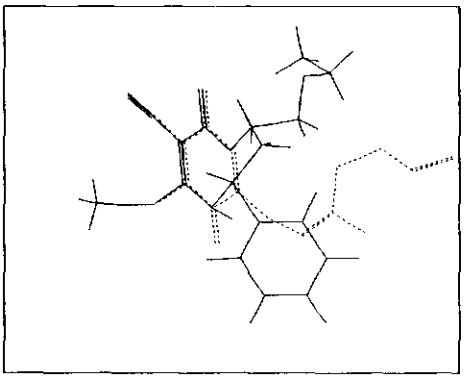
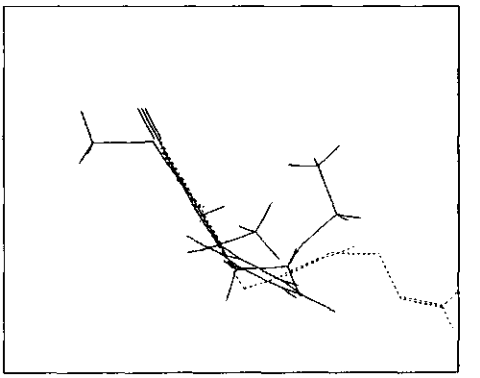




Ioxynil.





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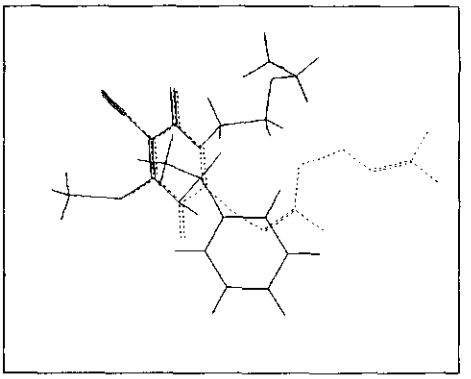

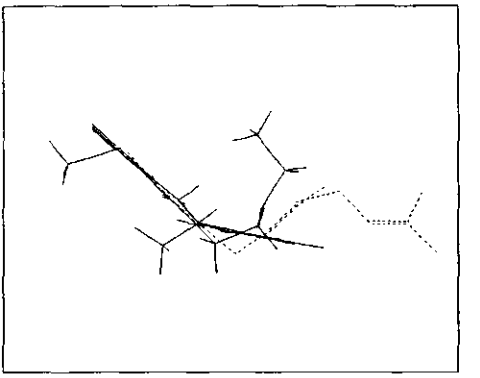

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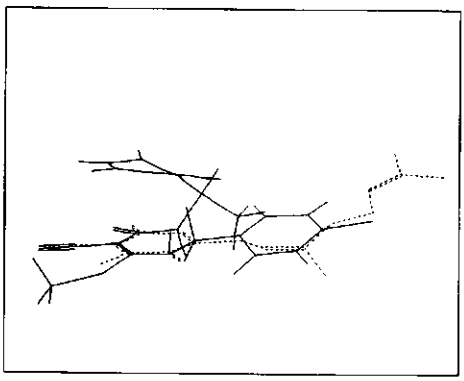

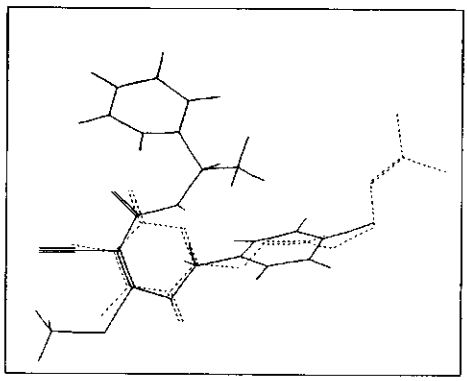

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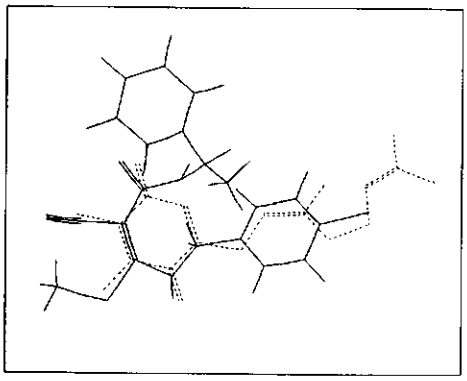

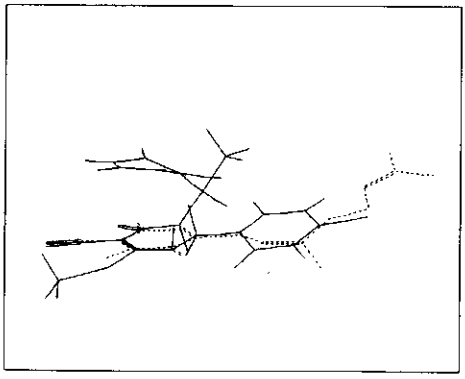

JNP-1223