Electrochromic absorbance changes in relation to electron transport and energy coupling in thylakoid membranes

Electrochrome absorptie veranderingen in relatie tot elektronen-transport en energiekoppeling in thylakoid membranen

Promotor: dr. W. J. Vredenberg,
hoogleraar in de plantenfysiologie,
met bijzondere aandacht voor de fysische aspekten

Jaap J. J. Ooms

Electrochromic absorbance changes in relation to electron transport and energy coupling in thylakoid membranes

Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op vrijdag 5 oktober 1990
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

The investigations were supported by the Netherlands Foundation for Chemical Research (SON), financed by the Netherlands Organization for the Advancement for Scientific Research (NWO) and performed at the Department Plant Physiological Research, Agricultural University Wageningen, The Netherlands.
The printing of this thesis was financially supported by the "LEB-Fonds".

Stellingen

1. De trage fase in het flitsgeïnduceerde P515-signaal in donkergeadapteerde chloroplasten, bestaat uit ten minste twee kinetisch te onderscheiden componenten.

   Dit proefschrift

2. De door Kramer en Crofts gebruikte methode om in vivo de aktiveringstoestand van het ATPase te bepalen uit het verval van het flitsgeïnduceerde P515-signaal, berust op de onjuiste veronderstelling dat dit signaal onder alle omstandigheden volledig bepaald wordt door een electrogeen verschijnsel.

   Kramer DM and Crofts AR (1989) Activation of the chloroplast ATPase measured by the electrochromic change in leaves of intact plants. Biochim Biophys Acta 976: 28-41.

   Dit proefschrift

3. Het middelen van flitsgeïnduceerde P515-signalen is alleen gerechtvaardigd indien gedurende het experiment de aktiveringstoestand van het ATPase niet verandert.

   Dit proefschrift

4. Sommige wetenschappers zijn zo gefascineerd door het computergebruik dat zij hun oorspronkelijke vraagstelling uit het oog verliezen.

5. Projecten voor gemeenschappelijk wonen zullen het inefficiënte gebruik van duurzame consumptiegoederen terugdringen.

6. Positieve discriminatie versterkt negatieve stereotypering van vrouwen.

7. De creativiteit die nodig is voor het oplossen van de milieuproblematiek komt meer tot uiting in het maken van slogans dan in een daadwerkelijke aanpak van het probleem.

8. Het idee dat de milieuproblematiek opgelost kan worden door economische groei, veronderstelt dat alles te koop is.

Brundtland GH (1987) Our common future. World commission on environment and development. Oxford University Press.

9. De bestrijding van het hondepoepprobleem is minder gebaat bij het trainen van honden dan bij het africhten van hun bazen.

Stellingen behorende bij het proefschrift "Electrochromic absorbance changes in relation to electron transport and energy coupling in thylakoid membranes" van Jaap Ooms.

Wageningen, 5 oktober 1990.

Voor mijn vader.

CONTENTS

Voorwoord

Op deze plaats wil ik graag een aantal mensen bedanken die in het bijzonder hebben bijgedragen aan het tot stand komen van dit proefschrift.
Wim Vredenberg, jouw kritische commentaar en enthousiaste manier van meedenken hebben mij erg gestimuleerd en ervoor gezorgd dat ik kritisch bleef op mijn resultaten en schrijfwerk.
Leen Peterse en Henk Melissen hebben steeds gezorgd voor voldoende kwalitatief hoogwaardig plantenmateriaal.
Wilma Versluis heeft vele "saaie" P515- en ATP-metingen met veel zorg uitgevoerd.
Pieter van Vliet heeft, in het kader van een doktoraalvak, met enthousiasme gedurende vijf maanden aan het onderzoek meegewerkt en daarmee de basis gelegd voor hoofdstuk 4.
Jan Snel, Olaf van Kooten en Dirk Naber wil ik bedanken voor de manier waarop zij mij wegwijs hebben gemaakt in de fotosynthese.
Zonder Willem Buurmeijers "absplot en efit" was het rekenen aan P515 signalen met meer problemen gepaard gegaan en waren sommige conclusies missschien helemaal niet mogelijk geweest.
Verder bedank ik iedereen die professioneel en/of vriendschappelijk heeft bijgedragen aan de goede sfeer op PFO en mijn werk heeft mogelijk gemaakt.
Tot slot Carla bedankt voor de omslagtekening en nog veel meer!

Abbreviations

ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
ATPase	thylakoid membrane H^+-ATP synthetase complex
a.u.	arbitrary units
BSA	bovine serum albumin
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CF_0	thylakoid coupling factor 0
CF_1	thylakoid coupling factor 1
cyt b_L	cytochrome b_{563} (low potential)
cyt b_H	cytochrome b_{563} (high potential)
DAD	diaminodurene
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCCD	dicyclohexylcarbodiimide
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DTE	1,4-dithioerythritol
DQH_2	durohydroquinone
EDTA	ethylene diamino tetra acetate
ETC	electron transport chain
Fd	ferredoxin
FeS	Rieske iron-sulfur protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LHC	light harvesting pigment complex
MES	2-(N-morpholino)-ethanesulfonic acid
MV	methyl-viologen
$NADP^+$	nicotinamide-adeninedinucleotide (oxidized)
NADPH	nicotinamide-adeninedinucleotide (reduced)
NQNO	2-n-nonyl-4-hydroxyquinoline N-oxide
P515	Absorbance change at 518 nm
P680	photosystem 2 reaction center
P700	photosystem 1 reaction center

Pheo	Pheophytin
pmf	proton motive force
PQ	plastoquinone
PQH_2	Plastoquinol
PS1	photosystem 1
PS2	photosystem 2
pBQ	1,4-benzoquinone
Q_A	primary quinone electron acceptor of photosystem 2
Q_B	secondary quinone electron acceptor of photosystem 2
tricine	N-(2-hydroxy-1,1-bis(hydroymethyl)ethyl) glycine
ttx	tentoxin

1 INTRODUCTION

Photosynthesis is the most important energy conserving process in nature. All our daily food depends on photosynthetic energy conservation and the abundantly used fossil fuels are also products of photosynthesis.

In higher plants photosynthesis takes place in particular cellular organelles, the chloroplasts. The chloroplasts are separated from the cytoplasm by two membranes forming the chloroplast envelope. Within the chloroplasts two compartments can be distinguished; the chloroplast stroma and the thylakoid lumen. The thylakoid lumen is surrounded by the extensively folded thylakoid membranes forming clustered flattened vesicles. The biosynthesis of carbohydrates from CO_2 and H_2O occurs in the chloroplast stroma, and is catalyzed by soluble enzymes. This biosynthesis does not directly depend on light, but depends on the supply of NADPH and ATP formed in light driven electron transport reactions which are bound to the thylakoid membranes.

Processes associated with these thylakoid membrane bound reactions are subject of this thesis. Only a brief description of the light driven photosynthetic electron transport, with emphasis on aspects important for the understanding of this thesis, will be given. For more detailed general reading on the photosynthetic electron transport, the reader is referred to Haehnel (1984), Ort (1986) and Andréasson and Vänngård (1988).

1.1 Linear electron transport

Figure 1.1 shows a schematic drawing of the four main, thylakoid bound, protein complexes. Three protein complexes form the electron transport chain; photosystem 2, photosystem 1 including their light harvesting complexes and the cytochrome b/f complex. Structure and

function of photosystem 2 are discussed by Van Gorkom (1985) and Velthuys (1987), whereas the photosystem 1 complex is reviewed by Malkin (1987) and Reilly and Nelson (1988). The fourth complex is the thylakoid membrane ATPase, which couples the transmembrane proton motive force to the synthesis of ATP (see Haraux 1986 for a review).

The light harvesting pigment-protein complexes LHC1 and LHC2 (not shown in figure 1.1) are associated with photosystem 1 and 2, respectively. These pigment-protein complexes provide an optimal structure to absorb photons and to transfer the energy of these photons to the photosynthetic reaction centers of the photosystems. For a more detailed discussion of the function and structure of the light harvesting complexes the reader is referred to Fork and Satoh (1986), Zuber (1987) and Anderson and Andersson (1988).

If a photon hits the photosystem 2 reaction center (P680), located at the lumenal side of the membrane, an electron is ejected from this center, and transferred to the primary electron acceptor, a pheophytin molecule (Pheo). The secondary electron acceptor, a bound quinone (Q_A), is situated at the stromal side of the thylakoid membrane. Thus the electron transfer causes a transmembrane charge separation. With Fe^{2+}/Fe^{3+} as a possible intermediate (Petrouleas and Diner 1986), the electron is transferred from Q_A to a second quinone, Q_B. Q_B stays in a semi-reduced state until it receives a second electron. Q_B is fully reduced by this second electron, and while taking up two protons from the chloroplast stroma it is liberated in the plastoquinol pool. Concurrently, the oxidized P680 is reduced in a four step mechanism (S-state model) which results in the oxidation of water (Andersson and Åkerlund 1987).

The linear electron transport proceeds further by binding plastoquinol to its cytochrome b/f binding-site at the lumenal side of the thylakoid membrane, the Q_o-site (quinol oxidation site, also termed Q_z- or Q_p-site Cramer et al. 1987, Rich et al. 1987). After excitation of the photosystem 1 reaction center (P700), the oxidized center is reduced by electrons transferred from plastoquinol via the iron-sulfur protein, cytochrome f and the copper containing protein plastocyanin (PC). The excited electron from the P700 reaction center is transferred, via some intermediate electron acceptors, to ferredoxin (Fd), situated at the

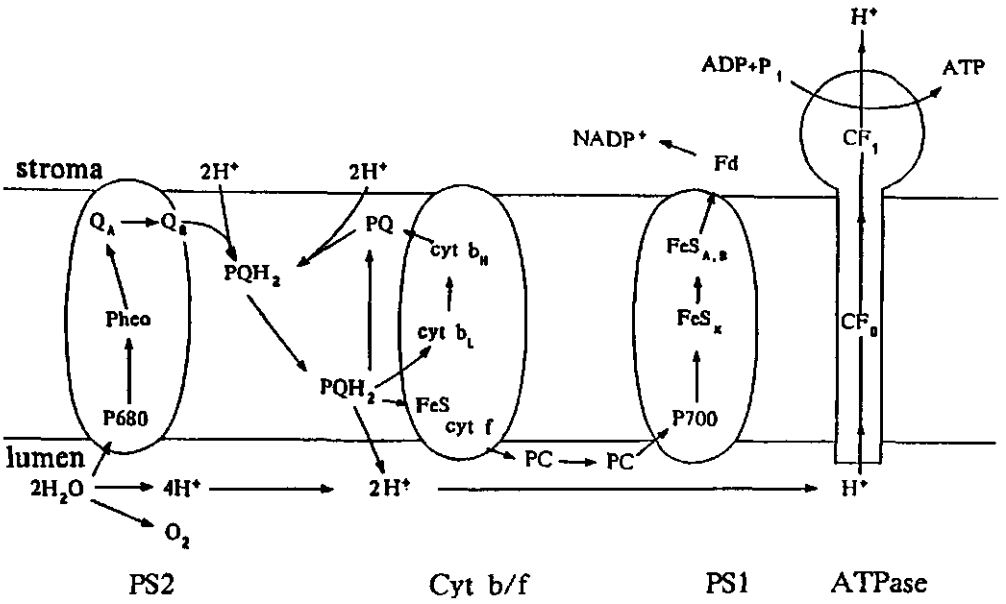

Figure 1.1. Schematic general representation of the photosynthetic electron transport chain. P680, reaction center of photosystem 2; Pheo, pheophytin, primary electronacceptor of photosystem 2; Q_A, secondary acceptor, a bound quinone; Q_B mobile quinone exchangeable with the plastoquinol pool; PQ, plastoquinone; PQH_2 reduced form of plastoquinone; FeS, Rieske iron sulfur center; PC, plastocyanin; P700, reaction center of photosystem 1; FeS_X, $FeS_{A,B}$, electron acceptors of photosystem 1; Fd, ferredoxin: cyt b_L and cyt b_H, cytochrome b_{563} low and high potential, respectively. See text for further details.

stromal side of the membrane (Malkin 1987). The first part of this electrontransfer, like in photosystem 2, results in a transmembrane charge separation. Ferredoxin can either reduce the ferredoxin-NADP $^+$ oxidoreductase finally resulting in NADPH in the chloroplast stroma, or it can induce cyclic electron transport involving cytochrome b/f, PQ-pool, plastocyanin and photosystem 1 (Carrillo and Vallejos 1987). Reduced ferredoxin is also involved in the thiol modulation reaction associated with the activation of the thylakoid membrane ATPase (Shahak 1982, Shahak 1985, Knaff 1989).

1.2 Q-cycle

Besides its function in the linear electron transport, the cytochrome b/f complex is suggested to function in a secondary electrogenic electron transport. Evidence for this suggestion came from measurements of unexpected high H^+/e^- ratios in linear electron transport (Hangarter et al. 1987, O'Keefe 1988) and from the observation of a slow component in electrochromic signals not associated with primary photochemistry (Joliot and Delosme 1974). The Q-cycle mechanism provides an explanation for this additional electrogenic charge separation. A plastoquinol binds to the Q_o-site of the cytochrome b/f complex, which is in contact with the lumenal phase of the thylakoid. Reduction of the iron-sulfur protein results in the oxidation of the plastoquinol to a plastosemiquinone which is further oxidized concomitantly with reduction of the low potential cytochrome b (cytochrome b_L) located at the lumenal side of the membrane. The electron is transferred to the high potential cytochrome b_H, which is situated near the stromal surface of the thylakoid membrane. The electrontransfer from cytochrome b_L to b_H has been shown to be at least partly responsible for a slow electrogenic charge separation (Jones and Whitmarsh 1987 and 1988). Accordingly, in plastoquinol oxidation one electron is transferred to the iron-sulfur protein and finally to PS1, whilst the other electron reduces cytochrome b. At the Q_r-site (quinone reduction site, also termed Q_c- Q_i- or Q_n-site Cramer et al. 1987, Rich et al. 1987), situated at the stromal side of the thylakoid membrane, a plastoquinone molecule oxidizes cytochrome b_H, and forms a plastosemiquinone anion. This semiquinone anion is further reduced either by a second turnover or by cyclic electron transport around PS1 involving reduced ferredoxin. The reduction of plastoquinone at the Q_r-site is allied with the net uptake of two protons from the stroma, which is partly responsible for the Q-cycle associated electrogenic process (Jones and Whitmarsh 1987 and 1988).

An alternative mechanism which accounts for the slow electrogenic phase and high H^+/e^- ratio is the b-cycle or modifications thereof (Wikström and Saraste 1984). In this model plastoquinol is oxidized at the Q_o-site; one electron is transferred to the iron-sulfur protein, and the second electron to cytochrome b_L which subsequently

reduces cytochrome b_H. In a second turnover plastoquinol reduces only the iron-sulfur protein, and the formed plastosemiquinone anion migrates in a special domain to the Q_r-site. At this site the plastosemiquinone oxidizes the cytochrome b_H, and forms a reduced plastoquinol again.

In modified models a transmembrane proton pumping is suggested to be coupled to redox changes in one of the electron carriers included in the cytochrome b/f complex (Joliot and Joliot 1986a and 1986b). More detailed discussions on different proposed mechanisms of the Q-cycle can be found in O'Keefe (1988), Cramer et al. (1987) and Rich and Moss (1987).

The common feature of all these mechanisms and the modifications thereof is a charge separation either by transmembrane electron- or proton movement. The occurrence of a slow rising phase in the flash-induced electrochromic absorbance change at 518 nm has been considered to be diagnostic for the operation of a Q-cycle (Cramer et al. 1987). However, it has been shown that under optimal and fully dark adapted conditions, when a Q-cycle is unlikely to be impaired by membrane energization, two kinetically different slow phases can be detected at 518 nm (Ooms et al. 1989). One component indeed is likely to be associated with a functional Q-cycle, while the other, more slowly rising and decaying component, is definetely non-electrogenic.

1.3 Proton translocation

As illustrated in figure 1.1 parallel with the light-induced electron transport, protons are accumulated in the thylakoid lumen. Two proton sources can be distinguished: i) protons released at the oxygen evolving complex (not shown in fig. 1.1) due to the oxidation of water and ii) protons released at the lumenal side of the membrane concomitant with the oxidation of plastoquinol at the Q_o-site.

The components of the photosynthetic electron transport chain are orderly but inhomogeneously distributed along the thylakoid membrane. The photosystem 2 protein complexes are mainly situated in the appressed granal membrane regions, whereas photosystem 1 and the ATPase are located in the stroma exposed thylakoid membranes

(Anderson and Andersson 1982). Only the cytochrome b/f complex is reported to be uniformly distributed in the thylakoid membrane (Albertsson 1985).

The ATPase couples the back-flow of protons accumulated in the thylakoid lumen during electron transport to the synthesis of ATP. The ATPase consists of a membrane embedded proton conducting part CF_0 and a water soluble nucleotide binding catalytic part CF_1. For a review on the structure and function of the chloroplast ATPase, see Haraux (1986). According to the chemiosmotic theory, the proton motive force (pmf) between the bulk aqueous phases separated by the membrane forms the driving force for ATP synthesis. Due to the lateral heterogeneity of the thylakoid membrane, continuous operation of the proton pumps and the ATPase needs a rapid equilibration of protons at the lumenal side and at the stromal side of the thylakoid membrane. Accordingly, coupling between redox reactions and ATP synthesis through fully delocalized proton flow in the bulk aqueous phases seems a reasonable assumption.

However, a number of experiments on electron transfer driven ATP synthesis does not agree with the chemiosmotic theory. These experiments have been interpreted to indicate localized proton motive force driven ATP synthesis. See Ferguson (1985), Melandri and Venturoli (1986) and Dilley et al. (1987) for reviews on this matter. Sigalat et al. (1985) found differential effects on the ATP synthesis rate if the pmf was attenuated either by lowering the light intensity or by nigericin. From the finding that these effects could be influenced by changes in the osmotic value of the assay medium, it was concluded that the degree of proton localization is related to the membrane stacking. The incomplete delocalization of protons might be explained by the proton resistance of the osmotic compartment itself (Sigalat et al. 1985). This is supported by the finding that alkalinization of the stromal partitition region between the appressed thylakoid membranes, is influenced by the stacking degree of the membrane. In unstacked chloroplasts the alkalinization is fast, whereas in stacked conditions the measured alkalization was found to be much slower (Polle and Junge 1986a). The slow alkalinization of the medium has been explained by hindered diffusion of protons and hydroxyl anions along the narrow and highly buffering gap between the appressed membranes (Junge and

Polle 1986).

Besides hindered diffusion of protons in the aqueous phase between tightly stacked membranes, sequestered proton domains have been suggested as an explanation for the experimental deviations from the original chemiosmotic theory (Dilley et al. 1987). It was found that chemical modification of neutral amines in the water oxidizing enzymes and the 8 kDa subunit of the CF_0 part of the ATPase were affected by protons from photosystem 2 water oxidation. In contrast protons released concomitantly with photosystem 1 plastoquinol oxidation did not affect the chemical modification (Prochaska and Dilley 1978b, Baker et al. 1981). These observations led to the postulation of sequestered proton domains, which were not accessible for protons released by photosystem 1. The existence of sequestered proton domains has been confirmed by experiments of other authors, and has been ascribed to amine buffering groups (Laszlo et al. 1984a, 1984b, Pfister and Homann 1986).

Further confirmation for the existence of sequestered proton domains came from the finding that low concentration of uncouplers greatly enhanced the inactivation of the water oxidizing enzyme by an alkaline medium pH (Theg et al. 1982). It was suggested that, in the absence of uncoupler, the medium pH was not directly sensed by the target site for photosystem 2 inactivation. Measurements of the absorbance change of neutral red indicating pH changes, showed that low concentration of uncoupler abolished the release of protons associated with water oxidation (Theg and Junge 1983, Polle and Junge 1986b). It was proposed that the uncoupler treatment depleted inner membrane proton buffering domains. As a consequence, the protons released upon the first flashes were supposed to be disposed in the depleted domains. After refilling of the domains, subsequent flashes caused a neutral red absorbance change associated with photosystem 2 proton release (Theg and Junge 1983). Additionally, evidence for localized proton domains has also been suggested from measurements of the effects of lipophilic tertiary amines on the pH dependent control of electron transport (Janowitz et al. 1990).

Recently a method was developed to shift the chloroplast energy coupling from localized to bulk phase delocalized (Beard and Dilley 1986, Beard and Dilley 1988a, 1988b, Beard et al. 1988).

Chloroplasts were stored either in a high salt or in a low salt medium. This differential storage resulted in different numbers of flashes needed for the onset of ATP synthesis and different effects of the permeable buffer pyridine on these flash numbers. The number of flashes needed for the onset of ATP synthesis was taken as indicative for the mode of coupling (localized or aqueous bulk delocalized) as well as for the protonation state of the proton domains (Dilley and Schreiber 1984, Theg et al. 1988). The availability of the technique to manipulate the coupling mode by changing the assay or chloroplasts storage medium might indicate the existence of elusive regulatory mechanisms for the coupling mode, and possibly resolve the contradictory experimental results on this subject (Beard and Dilley 1986). Nevertheless, it should be mentioned that the correctness of the methods used to manipulate the coupling mode and the interpretations of the experimental results are still under debate (Borchard and Junge 1990).

1.4 The electrochromic absorbance change at 518 nm

During light dependent photosynthetic electron transport in thylakoid membranes, an electric field is generated across the membrane. The membrane potential can be measured either directly with microelectrodes (Vredenberg and Tonk 1975, Van Kooten 1988), by extrinsic field indicating probes like oxonol VI (Krab et al. 1986) or by monitoring the electrochromic absorbance changes (Schapendonk and Vredenberg 1977, Junge and Jackson 1982). The flash-induced electrochromic absorbance change is generally accepted as a useful tool for studying the magnitude and kinetics of the formation and decay of the transmembrane electric field in photosynthetic membranes. An extensive review on this matter has been given by Witt (1979). The maximum of the electrochromic absorbance change is found at 518 nm and is generally termed the P515 signal. Assuming the presence of static innermembrane electric fields of high strength, the P515 absorbance change is likely to be linearly related to a homogenous transmembrane electric field (Witt 1979). However, thylakoids undergo drastic changes in their light scattering properties in response to electrochemical events. Consequently, the absorbance change at 518 nm is not suitable for use

under continuous illumination. Nevertheless it can be used as a measure of the transmembrane electrical potential in experiments using flash excitation with a low repetition rate (Junge and Jackson 1982, Crielaard et al. 1988) and with proper corrections for possible scattering changes.

The flash-induced rise of the P515 response in dark adapted algae and chloroplasts is known to consist of two phases. A fast one with a rise time of less than 0.5 ms and a slow rising phase (Joliot and Delosme 1974, Schapendonk et al. 1979). The fast phase is generally believed to reflect the primary charge separation in the reaction centers. In the terminology of Joliot and Delosme (1974), the fast phase is called phase A. In Schapendonk et al. (1979) it is attributed to the rising part of reaction 1. According to the analysis of Schapendonk et al. (1979), reaction 1, reflecting the primary charge separation in the reaction centers, reveals single exponential decay kinetics. Because of its origin in the reaction centers, reaction 1 will be termed in this thesis reaction 1/RC. The slow rising phase, phase B in Joliot and Delosme (1974) or referred to the rising part of reaction 2 in Schapendonk and Vredenberg (1979), has been subject of numerous discussions and interpretations.

It has been suggested that the slow rising part in the P515 response is due to charge delocalization associated with the electron transport linked release of H^+ and OH^- in the aqueous bulk phases (Olsen and Barber 1981, Zimányi and Garab 1982). Others have suggested a H^+-transporting Q-cycle or a modified version of a Q-cycle as an explanation for the slow component (Selak and Whitmarsh 1982, Jones and Whitmarsh 1985, Joliot and Joliot 1986b, Hope and Matthews 1987, Jones and Whitmarsh 1987). A cyclic electron flow around photosystem 1 has also been suggested to contribute to this slow phase (Crowther and Hind 1980, Shahak et al. 1980).

It is generally recognized that pre-illumination or a few shortly spaced pre-flashes accelerate the overall flash-induced P515 signal. This phenomenon resulting in biphasic decay kinetics, has been explained by the assumption that light activation of the thylakoid membrane ATPase leads to an enhanced proton conductivity through the CF_0 factor dissipating the transmembrane electric field (Girault and Galmiche 1978, Wise and Ort 1989, Kramer and Crofts 1989). However, as analyzed already by Schapendonk et al. (1979), the flash-induced P515

signal is a composite of at least two different responses, called reaction 1 (=1/RC) and reaction 2, respectively. They showed reaction 2 to be saturable with a few pre-illuminating flashes, resulting in an apparently accelerated decay of the P515 response due to enrichment of the signal with reaction 1/RC at higher flash numbers.

It has been argued (Schapendonk et al. 1979, Vredenberg 1981) that the dark recovery time of the slow component reaction 2, which is in general more than 500 ms, is too slow to originate from an electrogenic reaction. Therefore it has been suggested that reaction 2 is associated with the liberation and stabilization of protons in inner membrane domains (Schapendonk and Vredenberg 1979, Vredenberg 1981, Schreiber and Rienits 1982).

Crowther and Hind (1980) showed that the slow component associated with cyclic electron transport around photosystem 1 is different from reaction 2 as analyzed by Schapendonk and Vredenberg (1979). Recently it has been shown that the slow component in the flash-induced P515 signal is composed of at least two components, which can be distinguished in their rise and decay kinetics (Ooms et al. 1989, Ooms and Vredenberg 1989). One component referred to as reaction 1/Q is most probably associated with an electrogenic charge separation in a functional Q-cycle. Whereas the other component is reaction 2 as originally analyzed by Schapendonk and Vredenberg (1979). Due to its much slower rise and decay kinetics, reaction 2 is unlikely to be associated with an electrogenic reaction. Finally a very slow decaying component, originally termed phase d (Schapendonk et al. 1979), later called reaction 3 (Vredenberg et al. 1984), can be identified. Reaction 3 is non-electrochromic, non-electrogenic and gramicidin insensitive (Vredenberg et al. 1990).

Summarizing four kinetically different components can be distinguished in the flash-induced P515 signal. (i) Reaction 1/RC, resulting from the primary charge separation in the reaction centers, (ii) Reaction 1/Q, with slow rise kinetics, resulting from an additional charge separation in a functional Q-cycle. (iii) Reaction 2, with definitely slower rise and decay kinetics than reaction 1/RC and Reaction 1/Q, probably reflecting a non-electrogenic phenomenon. (iv) Reaction 3, which is gramicidin insensitive, non-electrogenic and non-electrochromic.

1.5 Outline of this study

The study presented in this thesis is carried out to obtain a more detailed insight in the magnitude and kinetics of the flash-induced P515 signal and the relation of these quantities to physiological processes of electron transport and energy conservation.

In chapter 3 intact dark adapted spinach chloroplasts were used, and the influences of chemicals interfering with the photosynthetic electron transport on the P515 signal were studied. It is shown that the slow rising phase in the P515 response in fact consists of two kinetically different components related to different membrane processes.

In chapter 4 the effects of an activated ATPase on the decay kinetics of the flash-induced P515 response were studied. Furthermore experiments on the relation between the extent of the non-electrogenic component, reaction 2 and the onset of ATP synthesis are presented.

Chapter 5 deals exclusively with the non-electrochromic and non-electrogenic component reaction 3. With aid of artificial donors, acceptors and inhibitors of the photosynthetic electron transport, the origin of reaction 3 could be located more precisely. However, the underlying mechanism of this reaction has not been elucidated.

Part of the results in this thesis have been published in Ooms et al. (1989), Ooms and Vredenberg (1989), Ooms et al. (1990a and 1990b), Vredenberg et al. (1990).

2 MATERIALS AND METHODS

2.1 Growth of Plants

Spinach

Spinach plants (*Spinacia oleracea* cv Amsterdams breedblad) were grown in a green house on a mixture of gardener soil and sand. In order to keep the light intensity above the 80 W/m^2, the plants received supplementary light from high pressure mercury lamps (Philips HPLR 400) during a light period of 8 hours. Heat radiation from the lamps was dissipated by a waterfilter of 7 cm thickness with a continuous flow of water. The temperature was kept at about 18 °C and the relative humidity of the atmosphere at 70%. For the isolation of chloroplasts, we used 4 weeks old plant material. Sometimes it was necessary to treat plants with insecticides. In those cases plants were not used for chloroplast isolation within a period of 4 days after these treatments.

Pea

Pea plants (*Pisum sativum* L. cv Finale) were grown in a climate chamber on a mixture of gardener soil and sand. The light intensity was 33 W/m^2, temperature 20 °C and relative humidity 70%. The light period was 14 hours. Chloroplasts were isolated from 2 weeks old seedlings.

2.2 Isolation of Chloroplasts

Spinach Chloroplasts

Spinach leaves (20 grams) were harvested in the morning after about 1 hour illumination. Routinely, the leaves were gently washed with tap water and demi-water, respectively. The isolation of chloroplasts is carried out under weak light intensities (max. 0.1 W/m^2) and on ice. The

veins were removed and the leaves were cut into small pieces. These pieces were brought into 50 ml grinding medium containing: 0.3 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4, 2 mM ascorbate, 4 mM cysteine and 50 mM MES/KOH pH 6.2. The leaf pieces were ground in this medium in a Sorvall Omnimixer with 2 subsequent 1 second pulses at maximum speed. The homogenate was filtered through three layers of nylon cloth. The filtered suspension was spinned down in two tubes in a MSE Chillspin centrifuge at 1000g for 55 seconds and the supernatant was carefully decanted. The top layer of the pellet, containing broken chloroplasts, was carefully washed off with 1 ml resuspension medium, containing: 0.3 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 0.2 mM KH_2PO_4, 1 mg/ml BSA and 50 mM HEPES/KOH pH 6.7 . Again 1 ml of resuspension medium was added to the pellets and by gently shaking the pellet was resuspended. Following resuspension the volume in both tubes was replenished to 25 ml. After centrifugation at 1000g for 55 seconds, the nearly clear supernatant was decanted and the pellet was carefully resuspended in a small volume of resuspension medium and was finally stored on ice. This procedure yielded preparations of 80-90% intact chloroplasts as determined by O_2-production with ferricyanide as electron acceptor (Heber and Santarius 1970). Total chlorophyll concentrations were determined according to the method of Bruinsma (1963).

Pea Chloroplasts

Isolation takes places on ice under weak light, maximum intensity 0.1 W/m^2. Freshly harvested leaves (about 20g) were mixed in a Sorvall Omnimixer by 3 subsequent 1 second pulses at maximum speed in 50 ml grinding medium, containing: 343 mM sorbitol, 0.4 mM KCl, 0.1 mM $MgCl_2$, 2 mM ascorbate, 4 mM cysteine and 50 mM HEPES/KOH pH 7.8. The suspension is filtered through 3 layers of nylon cloth and spinned down for 50 seconds at 1000g in a Chillspin centrifuge. Further procedures were as described above for the isolation of Spinach chloroplasts, except for the resuspension medium. Pea resuspension medium contained: 343 mM sorbitol, 5 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM KH_2PO_4, 2 mM ascorbate, 4 mM cysteine, 10 mg/ml BSA and 50 mM HEPES/KOH pH 7.8.

2.3 Flash-induced absorbance changes

In all flash-induced absorbance change measurements, we used a chloroplast concentration equivalent to 25 µg chlorophyll per ml in a final volume of 2 ml. For all experiments chloroplasts were freshly isolated. Experiments were done within 6 hours after isolation. All absorbance changes were measured with chloroplasts suspended in Basic reaction medium (medium B) of the following composition: 330 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 75 mM HEPES/KOH pH 7.5.
In chapter 4, measurements are reported which were performed in ATP-reaction medium (medium A), containing: 10 mM sorbitol, 3 mM $MgCl_2$, 1 mM KH_2PO_4 and 50 mM tricine/KOH pH 7.75 (see below).

2.3.1 P515 measurements

P515 measurements were carried out using a modified Aminco Chance spectrophotometer (Snel 1985). The modification provided a monochromatic measuring beam focused upon and passing through a 1x1x1.5 cm^3 suspension volume in the cuvette. The intensity of the measuring beam routinely was 43 mW/m^2 with a bandwidth of 12 nm. The cuvette was thermostated at 10 °C. Measuring light was provided by a tungsten lamp connected to a stabilized power supply (Oltronics B32-20R). Saturating actinic flashes were generated by Xenon flash tubes (General Electric FT-230) connected to a 8 µF capacitor giving a flash duration of 8 µs at half intensity; however a small tail of less than 1% of the total energy extended to about 150 µs. The flash reached the sample cuvette via a glassfiber lightguide, shielded by a Schott RG 630 or RG 645 filter. The photomultiplier was protected from the actinic light by a combination of 3 mm BG-39, Corning 4-96 and Wratten 40 filters. The photomultiplier signal was amplified after compensating the dark signal with a laboratory build amplifier (PFO-C140b). Further processing of the signals and generation of triggers for flashes was carried out with a minicomputer (Minc-11/23, Digital Equipment Corporation) as described by Snel (1985).

P515 measurements during and after continuous illumination, were carried out using an Aminco DW2A spectrophotometer in the dual wavelength mode at the wavelength pair 518-540 nm. The chloroplast suspension in the cuvette was gently stirred by a small magnetic stirrer. Continuous illumination from a 250 W halogen-tungsten lamp was guided to the cuvette by a glassfiber, shielded with a Schott RG 630 filter. The photomultiplier was shielded from actinic light with the same filter combination as used for the flash measurements. Absorbance changes were recorded on a mV pen recorder.

Flash-induced P515 responses were fitted as the sum of exponential functions, using the weighted least squares fitting procedure, yielding the best fit parameters (Press et al. 1988). All fits are corrected for the non-electrochromic gramicidin insensitive component, reaction 3 (Vredenberg et al. 1990) (see also chapter 3 and 5).

2.3.2 Cytochrome measurements

Flash-induced changes in the redox state of cytochrome f and cytochrome b were measured with the same equipment used for flash induced P515 measurements. The bandwidth of the measuring beam was reduced to 5 nm and the intensity to 20 mW/m^2. The redox changes of cytochrome f were taken as the difference of absorbance changes measured at 554 and 540 nm. For cytochrome b_{563} measurements the wavelength pair 563 and 575 nm was used. For the measurement of cytochrome b_{563}, the filter combination shielding the photomultiplier from the actinic light, was replaced by a combination of a Balzer K-55 and Corning 4-76 filter.

2.3.3 P700 measurement

Changes in the redox state of P700 were determined measuring absorbance changes at 830 nm. Measurements were carried out using a modified pulse modulation fluorometer (H. Walz, Germany) in the reflection mode as described by Schreiber et al. (1988). Repetitive 1 μs

measuring light pulses at a frequency of 100 kHz, were obtained from light emitting diodes (LED) at 830 nm. The signal was detected by a photodiode. Both the light emitting diodes and the photodetector were shielded by a Schott RG 780 cut-off filter, to prevent interferences with visible light. Light emitting diodes and photodetector were connected to the sample by a multi-branched light fiber. The top end this light fiber branch was positioned directly against the cuvette in the P515 measuring set-up, after removing the photomultiplier. To prevent the transmittance of light above 780 nm to the photodiode, the actinic light flashes were shielded by a combination of Balzer DT cyan and a Schott Calflex filter. This set-up ensures almost identical sample conditions as during P515 and cytochrome measurements.

2.4 Fluorescence measurements

Flash-induced fluorescence changes, were recorded using a PAM chlorophyll fluorometer (H. Walz, Germany) as described before (Schreiber 1986 and Schreiber et al. 1986). The sample cuvette is connected via a multi-branched glass fiber with a LED emitter, a photodiode detector and a xenon flash lamp. The cuvette is equipped with a rotating mirror on the bottom, providing full reflection of the fluorescence signal and keeping the chloroplast suspension homogeneous. The fluorescence signal is recorded on a Nicolet digital storage oscilloscope.

2.5 Flash-induced ATP formation

Flash-induced ATP formation was detected using the luciferin-luciferase ATP detection method with the LKB WALLAC ATP monitoring kit. The content of one vial is diluted in 10 ml distilled water and stored in 1 ml portions at -18 °C. Prior to each experiment the chloroplasts were broken in the cuvette. Unless stated otherwise we used the ATP-reaction medium (medium A, see before): 10 mM sorbitol, 3 mM $MgCl_2$, 1 mM KH_2PO_4 and 50 mM tricine/KOH pH 7.75, with further additions of: 5 mM DTE, 5 µM diadenosin pentaphosphate, 0.1

mM methyl viologen, 0.1 mM ADP and 200 µl ATP-monitoring kit (Beard and Dilley 1986). The chloroplasts concentration was equivalent to 25 µg/ml chlorophyll and the final assay volume was 2 ml. During the measurement the temperature was kept at 10 °C and the suspension was gently stirred. Actinic flashes were guided to the cuvette by a light fiber shielded by a Schott RG 645 filter. The photomultiplier was protected from actinic light by a combination of a Balzer K-55 and a Corning 4-76 filter. Changes in the bioluminescence were recorded on a mV recorder. After each measurement the system was calibrated by adding 0.2 nmol ATP from a stock solution.

3 EVIDENCE FOR AN ELECTROGENIC AND A NON-ELECTROGENIC COMPONENT IN THE SLOW PHASE OF THE P515 RESPONSE IN CHLOROPLASTS

3.1 Introduction

The flash-induced rise of the P515 response in dark adapted chloroplasts is known to consist of two phases. A fast one with a rise-time of less than 0.5 ms and a slow rising phase (Joliot and Delosme 1974). The fast phase is believed to reflect the primary charge separation in the reaction centers of the photosystems. In the terminology of Joliot and Delosme (1974) this phase is called phase A, in the experiments of Schapendonk et al. (1979) it is attributed to the rising part of reaction 1. We will refer to this reaction as reaction 1/RC because of its origin in the primary charge separation in the reaction centers.

The origin of the second slow rising phase, either called phase B (Joliot and Delosme 1974), or referred to as the rising part of the so called reaction 2 (Schapendonk and Vredenberg 1979) has been subject of numerous discussions and interpretations.

It has been argued (Schapendonk et al. 1979, Vredenberg 1981) that the dark recovery time of reaction 2, in general more than 500 ms, is too slow to be associated with an electrogenic reaction. Therefore it has been suggested that reaction 2 is associated with the liberation and stabilization of protons in inner membrane domains (Schapendonk and Vredenberg 1979, Vredenberg 1981, Schreiber and Rienits 1982). Also a cyclic electron flow around photosystem 1 has been suggested to contribute to this slow phase (Crowther and Hind 1980, Shahak et al. 1980). Others have suggested a H^+-transporting Q-cycle or a modified version of a Q-cycle as an explanation for the slow component (Selak and Whitmarsh 1982, Jones and Whitmarsh 1985, Joliot and Joliot 1986b, Hope and Matthews 1987, Jones and Whitmarsh 1987).

It has been shown (Crowther and Hind 1980) that the slow

component associated with cyclic electron transport around photosystem 1 is different from reaction 2 as analyzed by Schapendonk and Vredenberg (1979). Further indication for the existence of at least two different slow phases came from experiments using multiple flashes. The slow phase associated with reaction 2 is nearly completely suppressed after the first flash (Schapendonk et al. 1979, Schapendonk and Vredenberg 1979, Van Kooten et al. 1983), whereas reduction of cytochrome b was shown to be unaltered in the second flash (Van Kooten et al. 1983). This was taken as additional evidence that the slow component of the P515 response is not a reflection of an electrogenic Q-cycle.

Here we show that the so called slow phase in the P515 signal of intact chloroplasts is composed of two components. One component reflects the electrochromic effect of an electrogenic Q-cycle and is called reaction 1/Q. The other slow component is called reaction 2, as before (Schapendonk et al. 1979, Schapendonk and Vredenberg 1979). Reaction 2 is clearly distinguishable from reaction 1/Q by its much slower rise and decay kinetics. Because of its slow recovery kinetics, reaction 2 is unlikely to be associated with an electrogenic reaction.

3.2 The absorbance change induced by ATP hydrolysis

As documented in detail elsewhere (Peters et al. 1983), and as will be outlined below the decay of the overall P515 respons under ATP hydrolysing conditions is much faster. This faster decay has been evidenced to be due to the absence of reaction 2 in the flash-induced P515 response, rather than to an enhanced membrane conductance during ATP-hydrolysis. This evidence is, amongst others based on observations of Schreiber and Rienits (1982). They showed that addition of ATP to a chloroplast suspension with an activated ATPase resulted in an absorbance increase in the dark to a stationary level with a complementary loss of a light induced P515 component with a slow decay rate (i.e. reaction 2). Figure 3.1 illustrates the ATP induced dark absorbance change. Addition of 150 μM ATP to fully dark adapted chloroplasts (figure 3.1 upper trace), does not cause a change in the absorbance. However, after 34 seconds continuous illumination in the

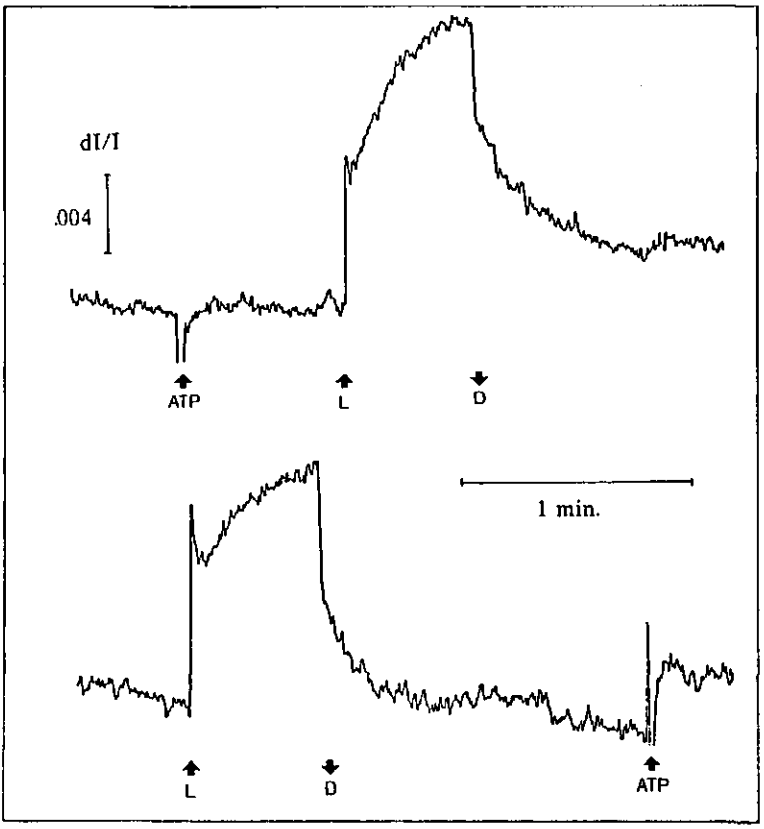

Figure 3.1. P515 response in continuous illumination and upon dark addition of ATP. Broken spinach chloroplasts were resuspended in 10 mM sorbitol, 3 mM $MgCl_2$, 1 mM KH_2PO_4, 5 mM DTE, 0.1 mM MV, 5 µM diadenosin pentaphosphate and 50 mM tricine/KOH pH 7.75. Arrows L and D indicate the switching on and off of continuous illumination, respectively. Arrow ATP, indicates the dark addition of 150 µM ATP.

presence of ATP, the absorbance change does not reverse to the original zero level. In contrast, after illumination in the absence of ATP, the absorbance level decays to the original dark level (figure 3.1 lower trace). After this pre-illumination, dark addition of ATP, results in a total absorbance change equal to the total absorbance change in figure 3.1 (upper trace). This experiment illustrates the "zero" level determined

under ATP hydrolysing conditions to be enhanced relative to the zero level determined under fully dark adapted conditions.

The experiment shown in figure 3.1 is in good agreement with the experiments of Schreiber and Rienits (1982). The faster decay of the flash-induced P515 signal under ATP hydrolysing conditions is ascribed to the saturation of the slow decaying component reaction 2 (Peters et al. 1983). Thus the flash-induced P515 response under ATP-hydrolysing conditions is a signal, enriched in other components (i.e. reaction 1/RC), superimposed on a higher stationary level of 515 nm absorbance (see figure 3.1).

3.3 The DQH_2 stimulated P515 signal

According to a current model (Selak and Whitmarsh 1982, Jones and Whitmarsh 1985, Rich 1988) one might expect either an induction or a stimulation of a Q-cycle by DQH_2. Consequently a complementary signal associated with this alteration will contribute to the P515 response. Figure 3.2 shows the P515 signal in dark adapted intact chloroplasts in the absence (curve A) and presence (curve B) of DQH_2, respectively. The difference between both signals (figure 3.2C) shows two effects of DQH_2. There is a significant transient stimulation of the P515 response upon each flash with relatively slow rise kinetics, and in addition there is an undershoot in the difference curve (see below).

The effect of DQH_2 on the absorbance change in broken chloroplasts with light-activated ATPase is shown in figure 3.3. The net effect of DQH_2 on the flash-induced absorbance change under ATP hydrolysing conditions is shown in figure 3.3C. In the absence (fig 3.2C and D) and presence (fig.3.3C) of an activated ATPase the DQH_2 stimulated signal shows a rise completed in about 40 ms and a recovery halftime of approximately 50 ms.

The DQH_2 induced undershoot of the difference signal in figure 3.2C might either be due to an increase in the decay of P515 components, or to a (partial) inhibition of one of these components. The slow recovery of the undershoot suggests the involvement of a slow P515 component. Measurements of the DQH_2 undershoot at other wavelength have confirmed that the spectrum is identical with P515.

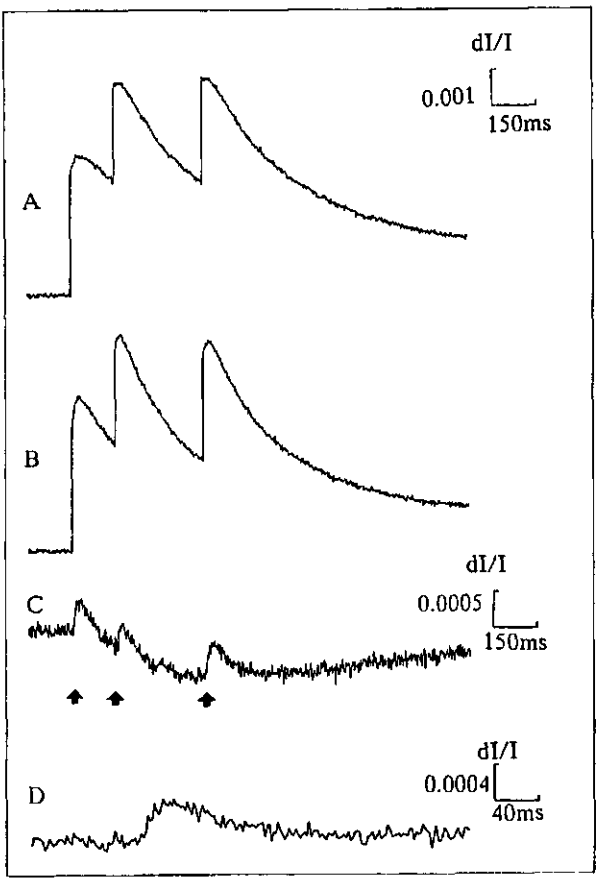

Figure 3.2. Flash-induced P515 absorbance changes in dark adapted intact spinach chloroplasts. Averages of five sweeps at a frequency of 0.05 Hz. Arrows indicate the time of the flash-firings. A: No additions. B: As A with 300 μM DQH_2. Trace C, difference curve, B-A, shows the effect of DQH_2 on the P515 response on an expanded scale. Trace D shows the effect of DQH_2 in the third flash on an expanded time scale.

Consequently, we conclude the very slow decaying non-electrochromic component reaction 3 not to be involved in the undershoot (Vredenberg et al. 1984, Vredenberg et al. 1990). Moreover it is shown that DQH_2 does not influence the amplitude or decay rate of reaction 3 (chapter 5). Thus a decrease of the amplitude or accelaration of the decay of

reaction 2 seems to be a feasible explanation for the undershoot.

Furthermore the undershoot in the DQH_2 difference signal is absent under conditions where reaction 2 is lacking due to saturation by ATP-hydrolysis (fig 3.3C). This gives further support for the suggestion that the undershoot is due to an inhibition of reaction 2. This inhibitory effect of DQH_2 on reaction 2 is further supported by the results of experiments in which reaction 2 has been suppressed by low concentrations of CCCP (see below). In the presence of CCCP there was little, if any, undershoot in the DQH_2 induced signal (not shown).

If it is assumed that DQH_2 does not affect the kinetics of existing P515 components, the difference signal in the third flash (figure 3.2D) can be considered as the net stimulation effect caused by DQH_2. This DQH_2 stimulated signal is attributed to reaction 1/Q.

The correctness of the assumption that DQH_2 at the concentration used does not affect the kinetics of P515 components is further justified by the following observations: i) DQH_2 was found to affect neither the amplitude nor the kinetics of the P515 signal in the presence of the PQ-antagonist DBMIB (not shown) ii) the kinetics of the DQH_2-stimulated signal are invariable irrespective of treatments which lead to large changes in the decay of the overall P515 signal due to modification of reaction 2 (see below). A faster decay for the P515 signal under reducing conditions (DQH_2) has been reported (Hope and Matthews 1987), which was proposed to be due to an increase in the membrane conductance. However, from our results it is clear that the apparent faster decay is due to partial inhibition of reaction 2.

3.4 The DBMIB sensitive P515 signal

Figure 3.3, trace D, E and F, show the effect of addition of 1 μM DBMIB on the P515 response under ATP hydrolysing conditions, i.e. under conditions where reaction 2 is saturated. Although small, DBMIB has a reproducible inhibitory effect on the signal (trace F=E-D). DBMIB is a plastoquinol antagonist and blocks the oxidation of plastoquinol and consequently a possible turnover of an electrogenic Q-cycle. Thus also under ATP hydrolysing conditions when the slow component reaction 2 is fully saturated, DBMIB achieved a small inhibition.

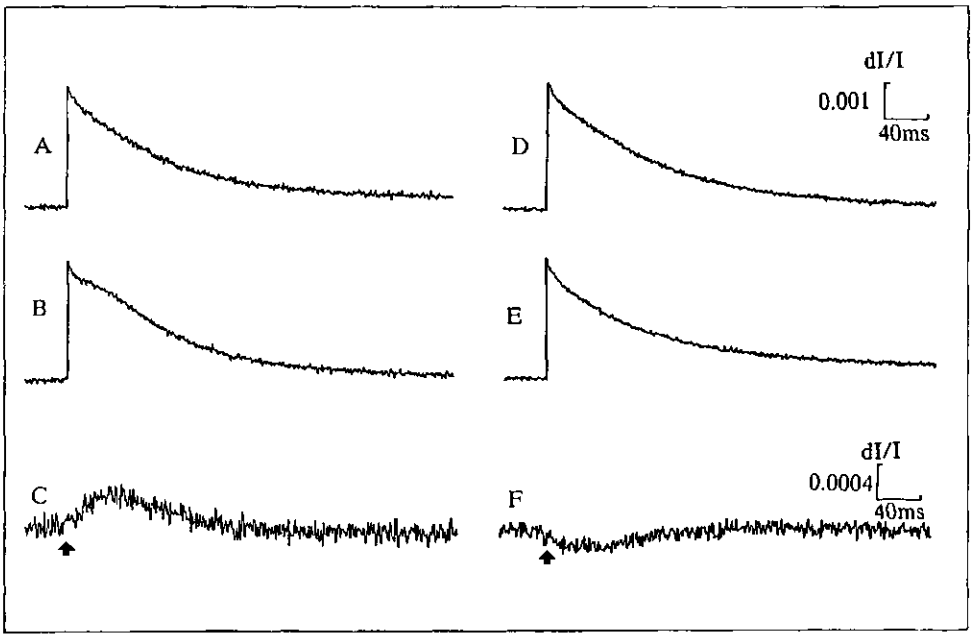

Figure 3.3. Flash-induced P515 signal at 15 °C in broken spinach chloroplasts, pre-illuminated (30s) in the presence of 300 μM ATP, 1 mM DTE, 60 μM MV and catalase (5000 units). Arrows indicate the time of the flash-firings. Curves are averages of 50 sweeps at a frequency 0.5 Hz. Two parallel experiments have been performed. Curves A and D were recorded 3 minutes after start of the pre-illumination. After the first recording, 300 μM DQH_2 (curve B) and 1 μM DBMIB (curve E) were added to the chloroplast suspensions. The second recording of 50 sweeps was started 7 minutes after the initial pre-illumination. Curve C shows the DQH_2 stimulated signal (curve B-A). Curve F shows the DBMIB inhibited signal (curve E-D).

The kinetics of the DBMIB inhibited signal under these conditions are hardly, if at all different from those for the DQH_2 stimulated signal (e.g. trace 3.3C). Consequently, under ATP hydrolysing conditions, which saturate reaction 2, a small endogenous Q-cycle contributes to the flash-induced P515 signal. Moreover in the presence of DBMIB, addition of DQH_2 has no effect on the signal at all (not shown), which means

that, as expected, DBMIB inhibits the DQH_2 stimulated signal.

A second method to saturate reaction 2 in the P515 response is the use of a train of a few saturating single turnover flashes. It has been shown (Schapendonk et al. 1979) that, after one flash reaction 2 is nearly completely saturated. This means that the absorbance change measured in the second flash is superimposed on a quasi-stationary absorbance level, caused by reaction 2 induced in the first flash.

Trace A and B in figure 3.4 show the absorbance change induced by two and one single turnover flashes, respectively. The same experiment in the presence of 1 μM DBMIB is shown in trace C and D, respectively. Trace E (=B-D) shows the net effect of DBMIB in a single first flash. Trace F (=A-B) and G (=C-D) show the net effect of the second flash in the absence and presence of DBMIB, respectively. In trace H (=F-G) the inhibitory effect of DBMIB in the second flash is shown. DBMIB inhibits a slow component even after reaction 2 has been eliminated (saturated) to a great extent by a pre-flash (e.g. fig. 3.4H). The kinetics of this DBMIB sensitive component are similar if not identical to those of the DQH_2 stimulated signals (figure 3.2D and 3.3C) and the DBMIB inhibited signal under ATP hydrolysing conditions (fig. 3.3F). Thus we conclude that the DBMIB-sensitive signal in secondary flashes shortly after a pre-flash reflects reaction 1/Q. The kinetics of curve E (fig. 3.4) as compared to curve H strongly suggests that DBMIB also causes a partial inhibition of reaction 2. We interprete curve E in figure 3.4 as reflecting a composite of reaction 1/Q and reaction 2 in response to inhibition by DBMIB. The inhibitory effect of DBMIB on reaction 2 has not been recognized and taken into account in the work of others (Selak and Whitmarsh 1982, Jones and Whitmarsh 1985, Hope and Matthews 1987, Jones and Whitmarsh 1987).

As discussed before (Vredenberg 1981), one would expect the recovery half time of a transmembrane electrogenic reaction induced by a Q-cycle to be the same as the half time for the transmembrane primary charge separation (reaction 1/RC). In our concept reaction 1/RC is exclusively measured after complete suppression of reaction 2 and reaction 1/Q. Thus the responses of figure 3.3A, 3.3D and 3.6C (see below), which show recovery half times of about 80 ms, are mainly if not exclusively caused by reaction 1/RC. Reaction 1/RC is known to decay in intact spinach chloroplasts with a half time of about 40-80 ms

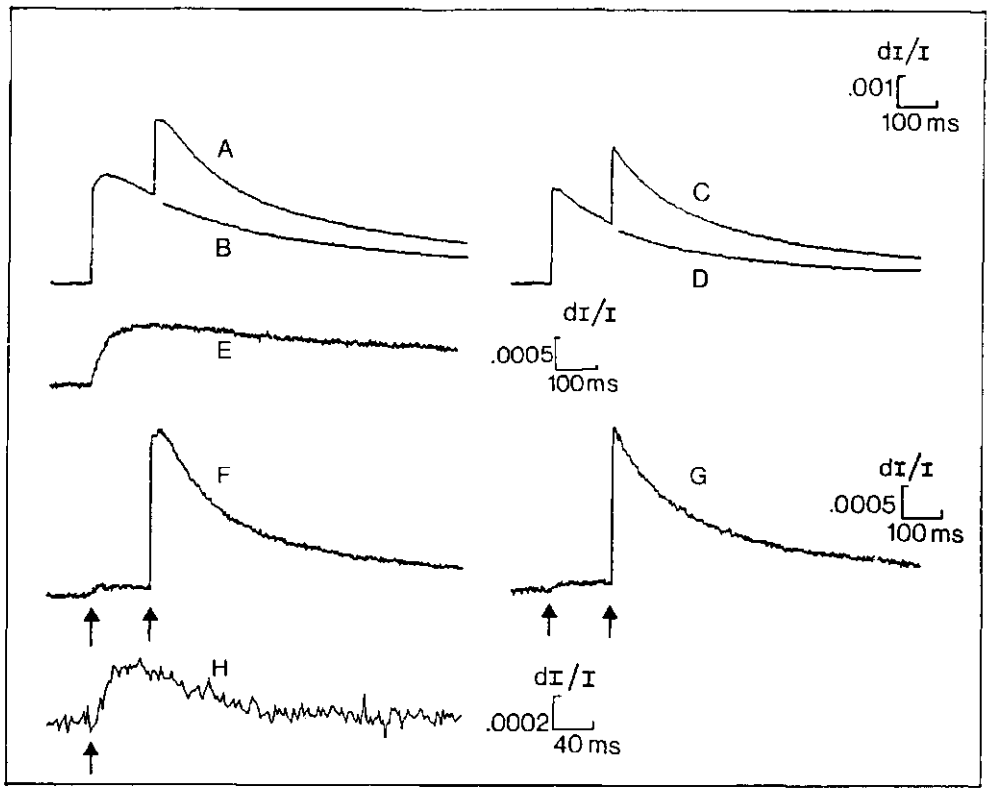

Figure 3.4. P515 response in intact spinach chloroplasts, upon one (B,D) and two (A,C) single turnover saturating flashes in the absence (A,B) and presence (C,D) of 1 μM DBMIB, respectively. Arrows indicate the time of the flash-firings. Curves are averages of 10 sweeps at a frequency of 0.05 Hz. Trace E (=B-D) illustrates the inhibitory effect of DBMIB in the first flash. Trace F (=A-B) and G (=C-D) represent the net effect caused by the second flash in the absence (F) and presence (G) of DBMIB. Trace H (=F-G) represents the effect of DBMIB on the response in the second flash.

(Schapendonk et al. 1979, Peters et al. 1983). The recovery half times found for the DQH_2 stimulated signal (figure 3.2D and 3.3C) as well as for the DBMIB inhibited signals (figure 3.3F and 3.4H) are of the same order as the recovery half times for reaction 1/RC indeed.

3.5 Cytochrome b turnover

In a functional Q-cycle electrons are transported across the membrane with cytochrome b as an intermediate. As a consequence each turnover of a Q-cycle giving rise to an associated P515 response (reaction 1/Q), should be accompanied by a turnover of cytochrome b. To avoid spectral contamination from electrochromic absorbance changes, measurements of cytochrome b turnover are generally performed in the presence of valinomycin. However, a transmembrane electric field and pH gradient have been reported to influence the turnover of a Q-cycle (Bouges Bocquet 1981). Because of this, Hope and Matthews (1987) discussed that the turnover of cytochrome b in the presence of valinomycin, should not be directly associated with the kinetics of the slow P515 signal. In contrast, recently Hope and Rich (1989) reported the transmembrane potential not to influence the proton uptake on the stromal side associated with quinone reduction by the cytochrome b/f reactions. This suggests no relevant effect of the trans-membrane potential on the turnover of a Q-cycle.

Being aware of the above mentioned objections, measurements of flash-induced cytochrome b turnover were accomplished in the presence of valinomycin and compared to P515 signals monitored under the same conditions omitting valinomycin. Figure 3.5A shows the flash-induced turnover of cytochrome b in dark adapted chloroplasts, with two consecutive flashes, both showing a turnover of cytochrome b. It is known that reaction 2 is strongly suppressed in the second flash of a 5 Hz flash-train (see for instance fig. 3.4 and Schapendonk et al. 1979), whereas reaction 1/Q is recurring in two or three consecutive flashes (fig.3.2).

The turnover of cytochrome b after light activation of the ATPase is shown in figure 3.5B. The kinetics of the cytochrome b turnover under these conditions differ hardly if at all from those in fig. 3.5A. Thus activation of the ATPase, which is known to saturate reaction 2 does not influence cytochrome b turnover.

Consequently with some prudence we interprete our results as an indication that the turnover of cytochrome b is only associated with reaction 1/Q. These results confirm the earlier conclusion of Van Kooten et al. (1983) that the slow phase of P515 caused by reaction 2 is

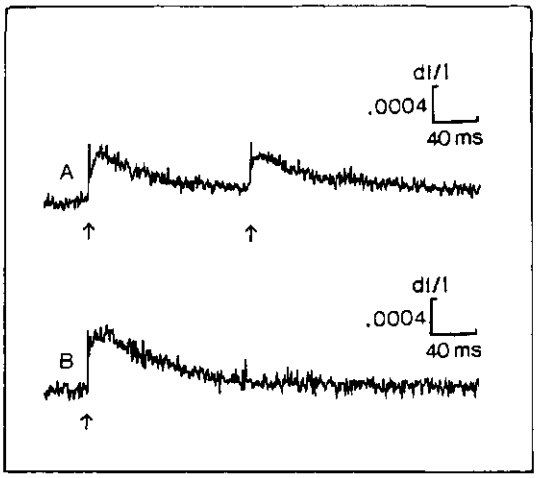

Figure 3.5. Turnover of cytochrome b, measured as the difference of absorbance changes at 563 nm and 575 nm in spinach chloroplasts. Arrows indicate the time of the flash-firings. Trace A: intact dark adapted chloroplasts, with 450 µM DQH_2, 60 µM methylviologen, 5000 units catalase and 1.5 µM valinomycin. Average of 100 sweeps at a frequency of 0.2 Hz. Trace B: chloroplast treatment as in figure 3.3 i.e. with 30 sec. pre-illumination and addition of 1.5 µM valinomycin. Average of 100 sweeps at a frequency of 0.5 Hz.

not associated with a turnover of cytochrome b.

The electrogenic reaction associated with a Q-cycle has been reported to be only partly inhibited by NQNO, while cytochrome b oxidation is fully inhibited by NQNO (Jones and Whitmarsh 1985, Jones and Whitmarsh 1988). It was concluded that the electrogenic reaction consists of two steps, the first associated with electron transfer between both b cytochromes, the second with cytochrome b oxidation. The electron transfer between the two b cytochromes is not detectable in our measurements, because both cytochromes have nearly the same absorption spectrum. Accordingly, from these experiments it is not possible to relate the kinetics of reduction and oxidation of cytochrome b with the kinetics of reaction 1/Q.

3.6 Reaction 2 suppression by CCCP

Figure 3.6A shows single flash responses of P515 in dark adapted chloroplasts, in the absence (A), after subsequent addition of 0,4 μM CCCP (B) and 0,4 μM CCCP + 1 μM DBMIB (C). CCCP substantially affects the overall P515 signal. This might be due to either an increase in the rate of decay of a P515 component, or to the inhibition of a component like reaction 2. It has been shown (Peters et al. 1984) that CCCP in the low concentration range (0.01-0.5 μM) causes a decrease in the extent of reaction 2 without affecting the decay rate of the response in repetitive flashes. The latter response is attributed to reaction 1/RC and has been reported to show enhanced decay rates at uncoupling CCCP concentration above 0.5 μM (Peters et al. 1984). It has been tested that at the CCCP concentrations used in the present experiment there was little, if any, effect on the decay rate of the response in repetitive flashes. Thus it is likely that, at the concentrations used, CCCP inhibits reaction 2. Further addition of DBMIB (trace C) illustrates a DBMIB sensitive P515 component after

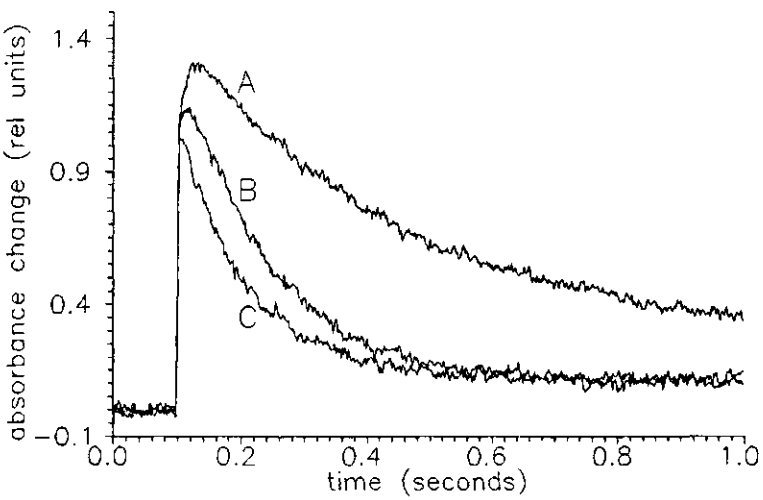

Figure 3.6. P515 response of intact spinach chloroplasts without any addition (A), with 0,4 μM CCCP (B) and with 0,4 μM CCCP + 1 μM DBMIB (C). Curves are averages of 20 sweeps at a frequency of 0.05 Hz.

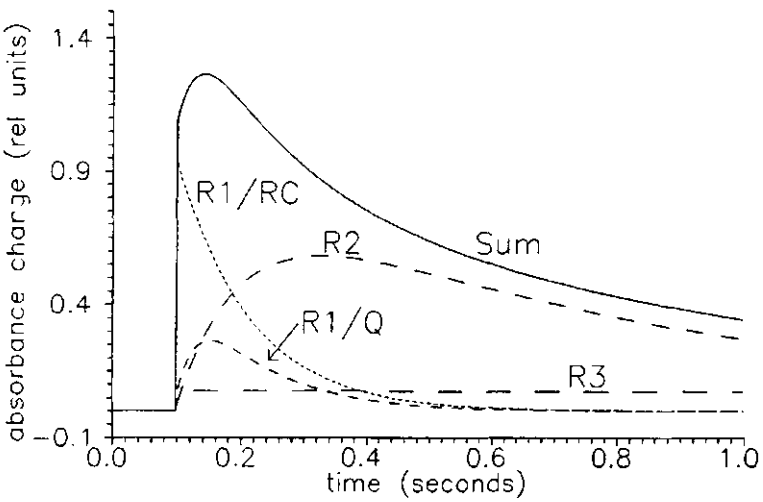

Figure 3.7. Illustration of the different flash-induced P515 components (see table 3.1 for fit parameters and text for more details). R 1/RC, reaction 1/RC originating from the primary charge separation. R1/Q, reaction 1/Q originating from charge separation in a functional Q-cycle. R2, reaction 2, non-electrogenic component possibly associated with innermembrane proton domains. R3, reaction 3 non-electrogenic and non-electrochromic absorbance change.

elimination of reaction 2 by CCCP. Figure 3.7 shows exponential fits of the difference signals. The CCCP inhibited component, trace A-B is attributed to reaction 2 (R2). The DBMIB sensitive signal after elimination of reaction 2, trace B-C is attributed to reaction 1/Q (R1/Q). The curves representing reaction 2 and reaction 1/Q are calculated according to the following general equation:

$$A_{(t)}=[A_{(0)}\{1-\exp(-k_r*t)\}]*\exp(-k_d*t)$$

The curves representing reaction 1/RC and reaction 3 are calculated from curve 3.6C according to the following general equation:

$$A_{(t)}=A_{(0)}*\exp(-k_d*t)$$

$A_{(0)}$ represents the maximal amplitude of the signal. The slowly rising components, reaction 1/Q and reaction 2 cannot reach this amplitude due to the concomitant decay of the signal. k_r and k_d represent the rise and decay constants, respectively. The calculated parameters are given in table 3.1.

Table 3.1. Fit parameters, for reaction 1/RC, reaction 1/Q and reaction 2 respectively as shown in figure 3.7. $A_{(0)}$ represents the maximal amplitude, k_r and k_d represent the exponential constants for the rise and decay, respectively and $t_{\frac{1}{2}}$ stands for the half rise or decay time, respectively.

		rise		decay	
	$A_{(0)}$	k_r	$t_{\frac{1}{2}}$(ms)	k_d	$t_{\frac{1}{2}}$(ms)
Reaction 1/RC	0.9	n.d.	n.d.	8.7	80
Reaction 1/Q	0.5	31.8	20	8.7	80
Reaction 2	0.9	10.2	70	1.3	530
Reaction 3	0.1	n.d.	n.d.	n.d.	n.d.

If the Q-cycle makes a full turnover upon each flash, the maximal amplitude of the Q-cycle related charge separation should be half of the amplitude associated with the primary charge separation in the reaction centers. This expected ratio between the amplitude of reaction 1/RC and reaction 1/Q is indeed confirmed by the calculated fit parameters of the experimental curves (table 3.1). Consequently this supports our view that solely reaction 1/Q should be ascribed to the operation of a functional Q-cycle.

Reaction 3 has not been taken into account sofar. It represents a non-electrogenic and non-electrochromic absorbance change, it is constant in the experiments presented here. Accordingly, in presenting

difference signals, reaction 3 is eliminated due to substraction. Reaction 3 will be discussed in detail in chapter 5.

3.7 Conclusions

We conclude that the component in the P515 response generally referred to as the slow phase in fact exists of an electrogenic and a non-electrogenic slow component which we call reaction 1/Q and reaction 2, respectively. Reaction 1/Q reflects the electrogenic charge separation, associated with a transmembrane Q-cycle. This conclusion is based on i) its on and off kinetics, ii) the amplitude which was determined to be half the amplitude associated with reaction 1/RC and iii) the sensitivity to DQH_2 and DBMIB. Reaction 2 is distinguished from reaction 1/Q with respect to its rise and decay kinetics, its saturable character and its sensitivity to DQH_2, DBMIB and ionophores. Reaction 1/Q in general is small as compared to reaction 2. This is the reason that is has mostly been overlooked so far under physiological conditions.

4 THE FLASH-INDUCED P515 SHIFT IN RELATION TO ATPase ACTIVITY IN CHLOROPLASTS

4.1 Introduction

It is generally recognized that the activation of the ATPase has a great influence on the decay kinetics of the P515 signal (Witt 1979, Morita et al. 1982, Peters et al. 1983). In recent literature (Wise and Ort 1989, Kramer and Crofts 1989) the overall decay rate of the P515 signal has been taken as a direct measure for the activation state of the thylakoid ATPase. However the slow rising component in the P515 absorbance change showing multiphasic rise and decay kinetics, is subject of different interpretations. Generally the slow rise is attributed to an additional transmembrane charge separation due to an electrogenic Q-cycle (Selak and Whitmarsh 1982, Hope and Matthews 1987, Jones and Whitmarsh 1988). In contrast we recently showed the slow rising phase in fully dark adapted chloroplasts to consist of two kinetically different components (see chapter 3) (Ooms et al. 1989). One component is electrogenic and due to an active Q-cycle. The other component is non-electrogenic; its decay is significantly slower than the decay associated with the transmembrane primary charge separation. This non-electrogenic component called reaction 2, has been suggested to be related with innermembrane proton domains (Schapendonk and Vredenberg 1979, Vredenberg 1981, Schreiber and Rienits 1982), possibly connected with the ATPase. Reaction 2 can be saturated, either by a few pre-flashes, a weak pre-illumination or by ATP hydrolysis (Ooms et al. 1989, Schapendonk and Vredenberg 1979, Vredenberg 1981). After a few flashes, the P515 signal is enriched in reaction 1/RC which is superimposed upon the saturated reaction 2 absorbance level, resulting in an apparent strong enhancement of the overall decay of the flash-induced P515 signal.

The apparent accelerated decay of the P515 signal under phosphorylating conditions is generally ascribed to an increased proton

conductivity through the ATPase. The decay of the overall P515 signal has been taken as a measure for the activation state of the ATPase. However in none of these studies (Girault and Galmiche 1978, Morita et al. 1982, Wise and Ort 1989, Kramer and Crofts 1989, Lemaire et al. 1985) the non-electrogenic slow component (reaction 2) has been taken into account. This may lead to serious misinterpretation of the decay kinetics of the flash-induced P515 signal and the activation state of the ATPase.

Here we present experiments on the kinetics of the flash-induced P515 signal in relation to the activation of the ATPase and the ATP synthesis during steady state. These experiments might contribute to a better understanding of the relation between the P515 decay kinetics, the onset of ATP synthesis and the steady state ATP synthesis rate.

4.2 Results

4.2.1 Flash-induced ATPase activity

Figure 4.1 shows two typical measurements of flash-induced ATP formation. The spikes on top of the signal are due to flash artifacts and can be used as flash markers. The measurement shown in trace A, is performed in medium A. The lower trace (B) shows a measurement performed in medium B, usually used for P515 measurements. The number of flashes given at the point where the first rise is detectable, is taken as the minimal number of flashes required to initiate ATP formation. We will term this the "Onset flash lag". The ATP flash yield is determined from the slope of the signal where a steady rise is reached.

It is shown that in medium B the onset of ATP formation is substantially delayed. We studied the number of flashes needed to initiate ATP synthesis in relation to the flash frequency. The onset flash lag in medium B is about 18, and is independent of the flash frequency within the range of 0.2-5.0 Hz (figure 4.2). In contrast, the steady state ATP production is decreased by lowering the flash frequency (figure 4.2). This experiment was repeated using reaction

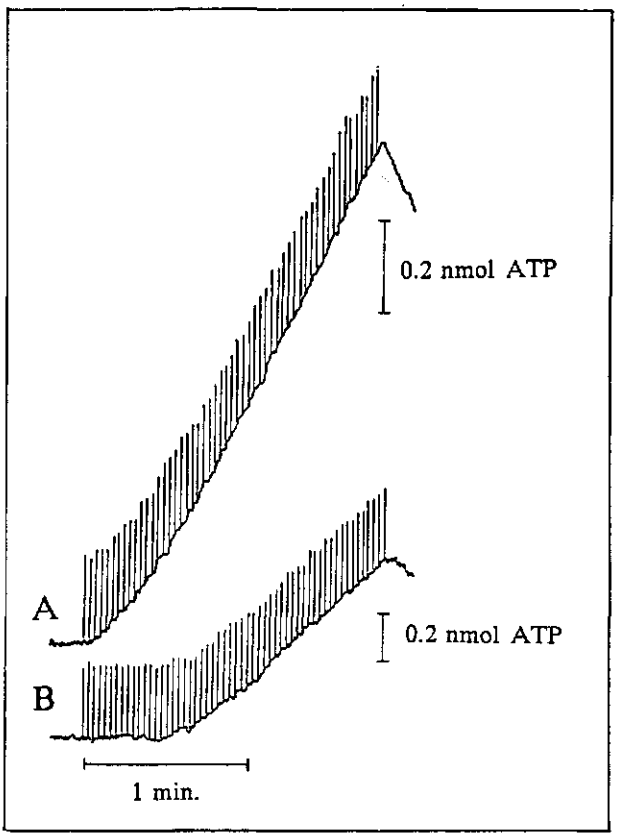

*Figure 4.1. Typical time recordings of flash-induced ATP formation. Curve A and B measured in reaction medium A and B, respectively. All further conditions as given in material and methods. Flash frequency 0.5 Hz. Steady state flash yield 0.53 and 0.46 nmol ATP/flash*mg Chl. for A and B, respectively. Note difference in ATP calibration bars.*

medium A and gave qualitatively the same results. The number of flashes required for the onset of ATP formation did not exceed three flashes and was also found to be independent of the flash frequency. The steady state ATP yield per flash was found to decrease at lower frequency similarly as in medium B.

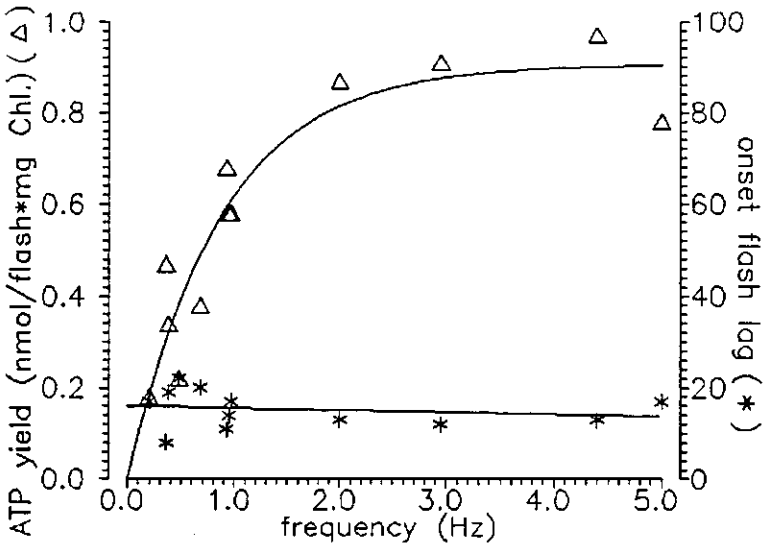

Figure 4.2. Flash frequency dependency of ATP yield and onset flash lag. Measurements performed in reaction medium B, further additions as indicated in material and methods.

Figure 4.3 shows the P515 signals upon the first flash in medium A and B, respectively. Fast and slow rising components, different in proportion, are clearly distinguishable in both responses. In medium B the overall decay of the P515 signal is accelerated. The fast rise in the P515 signal, equal in both media, is attributed to the transmembrane primary charge separation, reaction 1/RC (Ooms et al. 1989). The slow component associated with the Q-cycle (reaction 1/Q), can be neglected here, because under the experimental conditions used, its contribution is only small and consequently does not significantly influence the overall P515 signal (Ooms et al. 1989). As has been shown before (Ooms et al. 1989, Schapendonk and Vredenberg 1979) and as illustrated in figure 4.7 (see below), the slow rising component reaction 2 can be saturated by a few shortly spaced flashes. Thus upon following flashes the initial decay of the overall response is not mixed with a slow rising phase of reaction 2 any more, and is exclusively determined by the decay of reaction 1/RC. Consequently after a few shortly spaced

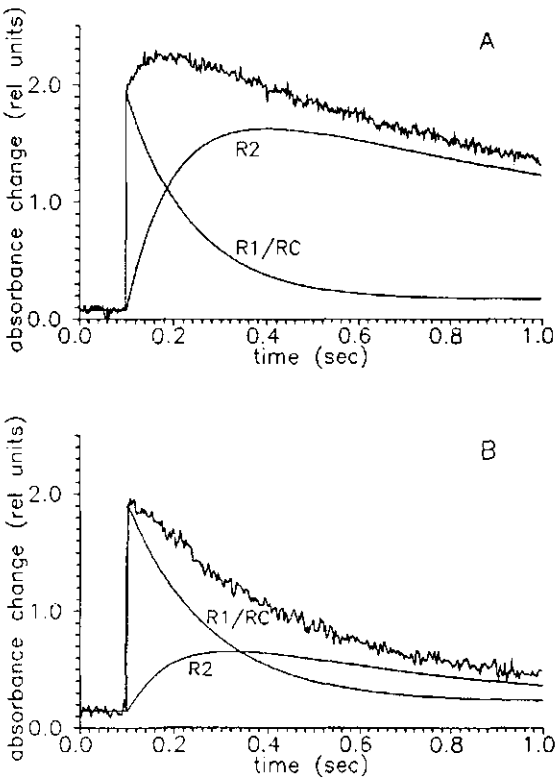

Figure 4.3. Flash-induced P515 signal upon a single sweep and flash in dark adapted chloroplasts. Measurement in reaction medium A (A) and B (B), respectively. Smooth figures are fitted curves for reaction 1/RC and reaction 2, respectively. See text for further details.

saturating flashes the kinetics of reaction 1/RC can be determined by analysis of the decay of the P515 signal. This analysis was performed in separate experiments for both media. The decay constants for reaction 1/RC were 7.2 and 5.8 in medium A and B, respectively (curves 1/RC in figure 4.3A and B). Consequently the great difference in the overall P515 signal in both media is due to a substantially lower extent of the slow component, reaction 2, in medium B. This lower extent of reaction 2, coincides qualitatively with a longer onset flash lag in medium B (figure 4.1).

4.2.2 The effect of CCCP

Addition of low concentrations of CCCP, up to 0.4 μM, increases the onset flash lag, but has no influence on the steady state ATP production (table 4.1). Lowering the flash frequency in the presence of CCCP, reveals that the onset flash lag is no longer frequency independent (figure 4.4). The steady state flash-induced ATP production in the presence of these concentrations of CCCP is frequency dependent and shows the same frequency profile as the control (e.g. figure 4.4, table 4.1). In addition, CCCP at the concentrations used strongly inhibits the slow component reaction 2 in the P515 signal, without affecting the decay of the transmembrane component reaction 1/RC, (figure 4.5). This effect of CCCP has been extensively documented before (Peters et al. 1984).

Table 4.1. Effect of addition of CCCP on the onset flash lag and the steady state ATP yield. Measurements performed in reaction medium A, with further additions as indicated in material and methods. Flash frequency 1Hz.

Concentration CCCP (μM)	onset flash lag	ATP yield nmol/flash*mg Chl.
0	3	0.82
0.1	7	0.86
0.2	9	0.84
0.3	15	0.84
0.4	21	0.84

Recently it was shown possible to manipulate the coupling between the electron transport and ATP synthesis by changing the salt concentrations in the chloroplast storage medium (Beard and Dilley 1986, Theg et al. 1988). We repeated these experiments, and measured the onset flash lag of the differentially stored chloroplasts in reaction medium A.

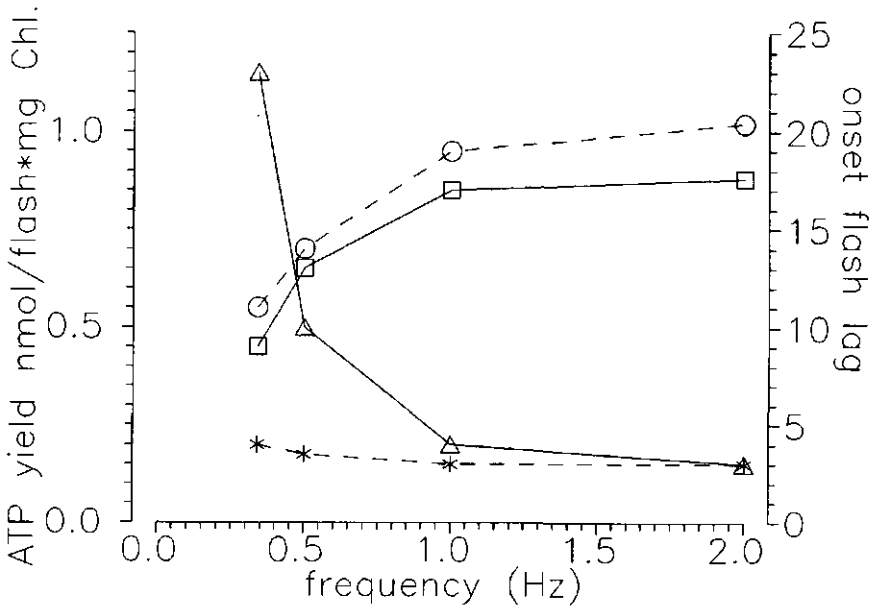

Figure 4.4. The ATP yield (o,□) and the onset flash lag(Δ,) as a function of the flash frequency in the absence (o,*) and presence (Δ,□) of 0.3 μM CCCP, respectively. Measurements performed in assay medium A, further additions as indicated in material and methods.*

We found an onset flash lag of 4 and 17 flashes in low (200 mM sorbitol) and high (100 mM KCl) salt stored chloroplasts, respectively (not shown). The P515 signals measured under the same conditions, revealed a suppression, in magnitude variable for different preparations, of reaction 2 in high salt stored chloroplasts (not shown, but see figure 4.3B and 4.5B for comparable signals). Thus the effect of storage in high salt medium, is qualitatively the same as addition of low concentrations of CCCP to the chloroplast preparation as used in our experiments (table 4.1 and figure 4.5).

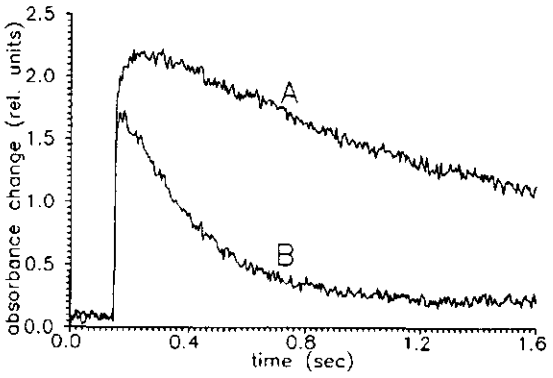

Figure 4.5. Single sweep flash-induced P515 signal in dark adapted chloroplasts. Measurement in reaction medium A, with additions as indicated in material and methods. Curve A, control; curve B, in the presence of 0.3 μM CCCP.

4.2.3 The P515 signal during steady state ATP production

After about 30-40 flashes in a 1 Hz flash train, the ATP-synthesis reached a constant rate (figure 4.1). We suppose that under these conditions the ATPase has reached its maximal activation state. In parallel experiments we measured the developing ATPase activity in a 1 Hz flash train and the P515 signals in the 60^{th} flash. Figure 4.6 shows the single exponential fits of the initial fast P515 component upon the 60^{th} flash, obtained after correction for the (slow) decay of the reaction 2 component saturated in the foregoing flashes (see for the overall decay in a similar experiment but after 10 flashes, figure 4.7). Curve 4.6A shows the signal in the presence of ADP, curve 4.6B in the presence of ADP and 25 μM DCCD. Curve 4.6C represents the response as determined from an independent experiment using multiple flashes at 5 Hz frequency in the absence of DTE and ADP (Schapendonk and Vredenberg 1979) (see also figure 4.7A for an illustration). DCCD is a blocker of CF_0 (Nelson-Sigrist et al. 1978). At the concentration of DCCD used here, the ATP synthesis rate was determined to be 60% of the control value. Unfortunately it was not possible to use higher concentrations of DCCD, to obtain a stronger inhibition of the ATPase.

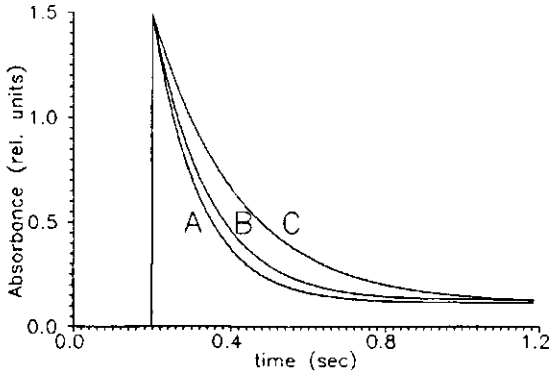

Figure 4.6. Exponential fits of flash-induced P515 signals, after correction for reaction 2 (see text for details). Measurements during ATP synthesis in reaction medium B, further additions as indicated in material and methods. Curve A, response upon the 60th flash from a 1 Hz flash train in the absence of DCCD. Curve B, the same as A but in the presence of 25 µM DCCD. Steady state ATP synthesis for B was 60% of control A. Curve A and B are averaged fits of 3 separate experiments. Curve C, reaction 1/RC as determined from a separate multi-flash experiment in the absence of ADP and DTE (see text for further details).

We found higher DCCD concentrations to cause an accelaration of the P515 signal under control conditions i.e. in the absence of ADP and DTE (Jahns and Junge 1989). Because of the correction for the contribution of reaction 2, the curves in figure 4.6 are those of reaction 1/RC, and represent exclusively the rise and decay of the transmembrane electric field. Two pathways for back flow of the transmembrane charge are generally recognized (Junge et al. 1970, Junge 1987); the passive dissipation through the membrane and secondly the proton conduction through the ATPase. This leads to the following equation for the time pattern of the electrogenic P515 signal:

$$A_{(t)}=A_{(0)}*\exp[-(k_1+k_{atp})*t]$$

where $A_{(0)}$ is the amplitude at t=0. k_1 and k_{atp} are determined by the passive membrane leakage and the proton conductivity of the

membrane ATPase, respectively. The sum of k_1 and k_{atp} represents the total decay constant. We assume k_1 to be independent of the ATPase activity. We further assume the proton conductivity through the ATPase linearly related to the ATP production rate. Thus according to the ATP measurements k_{atp} in curve B is about 60 % of its maximum value in curve A. Estimation of the value of the total decay constants (k_1+k_{atp}) from the curves shown in figure 4.6, then leads to the calculated values of k_1 and k_{atp} as shown in table 4.2.

The decay constant of reaction 1/RC, determined in a separate experiment under conditions where no ATPase activity is detected, (curve 4.6C) is in good agreement with the value for k_1, as calculated from curves A and B. This experiment reconfirms the transmembrane charge dissipation to be accelerated by an enhanced proton conductivity associated with ATP synthesis. The experiment is essentially similar, except for corrections of the non-electrogenic components, to the classical experiment of Junge et al. (1970) showing an enhanced P515 decay in the presence of ADP and P_i. Taken together it supports our view that reaction 1/RC exclusively monitors the transmembrane electric field.

Table 4.2. Decay constants for the curves shown in figure 4.6. The total decay constant (k_1+k_{atp}) for curve A and B are averaged from 3 separate experiments. k_1 and k_{atp} are calculated using the assumptions mentioned in text. k_1 is determined by passive membrane leakage. k_{atp} is attributed to proton conductance through the ATPase.

	ATP synth. rate	k_1+k_{atp} (s^{-1})	k_1 (s^{-1})	k_{atp} (s^{-1})
+ADP (A)	100 %	8.6 ± 0.5	5.0	3.6
+ADP+DCCD (B)	60 %	7.2 ± 0.9	5.0	2.2
control (C)	0 %	4.6	4.6	0

4.2.4 The P515 signal during flash ATPase activation

We further analyzed the P515 signal in medium A under different conditions in single sweeps of 10 flashes (5 Hz). Figure 4.7 shows the P515 signal under control conditions (A), in the presence of 0.1 mM ADP (B) and in the presence of 0.15 mM ATP (C), respectively. The amplitude and decay of the P515 signal in the control measurement and in the presence of ADP, after the first flash are equal (only partly shown). However after the second flash, the decay of the signal is accelerated in the presence of ADP (figure 4.7B). This coincides with the onset of ATP production in the second or third flash under the conditions used.

The P515 signal in the presence of ATP shows already in the first flash an accelerated decay relative to the control experiment. After the tenth flash, the signal measured in the presence of ATP does not decay towards the original zero level, but remains on a higher level. Analysis of the decay kinetics after the tenth flash gives the results shown in table 4.3.

It illustrates the amplitude of reaction 1/RC to be nearly constant under the different conditions. The decay of reaction 1/RC is accelerated in the presence of ADP. In the presence of 0.15 mM ATP, when hydrolysis takes place, the total decay constant (k_1+k_{atp}) is not significantly different from the control measurement. Obviously the expected enhanced proton conductivity, due to the activation state of the ATPase, does not result in an accelerated decay of reaction 1/RC. Probably the enhanced proton conductivity is compensated by a continuous proton influx resulting from ATP hydrolysis. Thus under these conditions, the decay of reaction 1/RC is solely determined by the passive leak through the membrane. Table 4.3 (line 4) shows the results of an experiment conducted under the same conditions as in figure 4.7. The decay of reaction 1/RC in the presence of the CF_1 antagonist tentoxin (Arntzen 1972) is inhibited compared to the decay under fully activated conditions (compare line 2 and 4 in table 4.3). If we adopt the assumptions made for the calculation on the DCCD inhibition in figure 4.6, the results in table 4.3 reveal a 72% inhibition of the ATPase. This result is in good agreement with the actually measured inhibition of the ATP synthesis rate of 79%.

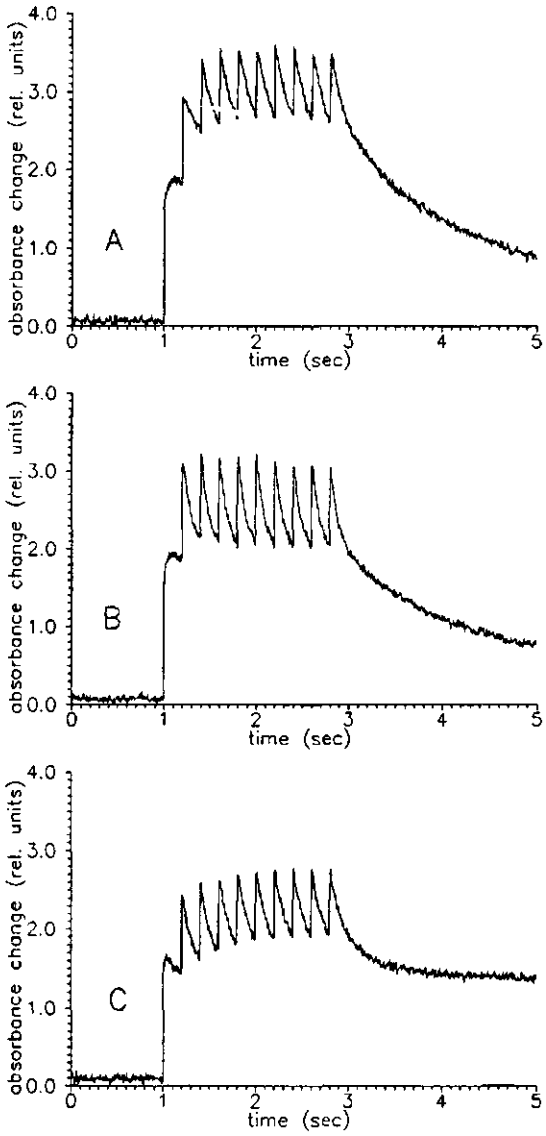

Figure 4.7. Multi-flash P515 signal from a single sweep flash train measured in reaction medium A. Further additions as for ATP formation measurements (see chapter 2). Curve A, control in the absence of DTE and ADP. Curve B, with addition of DTE and ADP. Curve C, with addition of DTE and ATP.

Although there is a clear tendency to a lower amplitude of reaction 2 in the presence of ADP, relative to its amplitude in the absence of ADP, this has not been definetely established (table 4.3). In the presence of ATP the amplitude of reaction 2 is lower and its decay is substantially inhibited. Schreiber and Rienits, (1982) showed that dark addition of ATP to a chloroplast suspension with preactivated ATPase, results in a dark absorbance change at 515 nm to a stationary level (see also figure 3.1). The result shown in figure 4.7C is in excellent agreement with these observations and illustrates the slow component in the P515 signal to be activated and saturable by ATP hydrolysis.

Table 4.3. Fit parameters of exponential fits from P515 signals measured under the conditions as in figure 4.7. Fits were made from the time point of the tenth flash. A1 and A2 amplitude of reaction 1/RC and reaction 2, respectively. k_1 *(*s^{-1}*) and* k_{atp} *(*s^{-1}*) as in table 4.2.* k_2 *(*s^{-1}*) represents the decay constant of reaction 2. Parameters of the "control" and "+ADP" measurements are averaged from 5 separate experiments. All other parameters are determined from a single experiment. Concentration tentoxin (ttx) 10 μM.*

	Reaction 1/RC				Reaction 2	
	A1	k_1+k_{atp}	k_1	k_{atp}	A2	k_2
Control	1.0 ± 0.2	6.4 ± 0.8	6.4	0	2.4 ± 0.6	0.7 ± 0.1
+ADP	1.1 ± 0.2	12.1 ± 1.5	6.4	5.7	1.7 ± 0.4	0.9 ± 0.3
+ATP	1.0	5.9	5.9	0	1.1	0.1
ADP+ttx	1.2	8.1	6.4	1.6	2.4	0.7

4.3 Discussion

The activation of the ATPase as well as the steady state ATP synthesis needs membrane energization (Junesch and Gräber 1987). The differential dependency of the onset flash lag and the steady state ATP flash yield on the frequency, suggests different mechanisms for the accumulation of flash induced proton motive force (pmf). Obviously the dark period between flashes has no influence on the onset flash lag, indicating the absence of an appreciable dark dissipation of energy required for the activation of the ATPase. In contrast, the flash frequency dependency of the steady state ATP flash yield, suggests a considerable leak of energy required for ATP synthesis in the dark period between flashes.

From P515 measurements (figure 4.7, table 4.3) it is clear that, under the experimental conditions used, the transmembrane electric field, sensed by reaction 1/RC, decays with a half time of about 110 ms. Thus in the frequency range 0.2-5 Hz it seems inappropriate to expect the transmembrane electric potential to be responsible for the flash frequency independency of the onset flash lag. Using attenuated flashes in a system where the electric component of the pmf is eliminated by the addition of nonactin, similar results were found (Hangarter and Ort 1986). It seems reasonable to conclude that a stable proton gradient is responsible for the flash frequency independency of the onset flash lag. Several authors reported on the existence and function of proton domains, which are not in equilibrium with the bulk aqueous phase, involved in the activation of the ATPase (Beard and Dilley 1986, Theg et al. 1988, Theg and Junge 1983, Hangarter and Ort 1985).

As shown in table 4.1 and figure 4.4, low concentrations of CCCP increased the onset flash lag. Moreover, in the presence of CCCP the onset flash lag is no longer frequency independent. However CCCP concentrations up to 0.4 μM, did not decrease the ATP flash yield during steady state (table 4.1 and figure 4.4), but caused an increase in decay of the overall P515 signal (figure 4.5). In the experiment presented in table 4.1, we used a flash frequency of 1 Hz, whereas the maximum flash yield is only reached at about 2 Hz (figure 4.2). We expect the lower ATP yield at lower flash frequencies to be due to passive membrane leaks. If CCCP induces a membrane leak competing

with the ATPase for protons, one would expect a decreased ATP yield per flash in the presence of CCCP. Thus the apparent acceleration of the P515 signal by low concentrations of CCCP, is most probably due to the inhibition of the slow component, reaction 2, and is not caused by an enhanced transmembrane proton conductivity.

Under phosphorylating, as well as under non-phosphorylating conditions, the initial decay of the flash-induced P515 signal seems to be accelerated after a few shortly spaced flashes (figure 4.7). In our interpretation, the apparent accelerated decay is due to the saturation of the non-electrogenic component reaction 2 (Ooms et al. 1989, Schapendonk and Vredenberg 1979, Vredenberg 1981). Consequently, the slow rise of reaction 2 does not contribute to the initial decay of the P515 signal (figure 4.7), and the curve fitting can be done properly without further assumptions. The experiments shown in figure 4.6 and 4.7 and table 4.2 and 4.3 demonstrate the decay constant of reaction 1/RC to be related to the activation state of the ATPase. It is also demonstrated that the decay of the non-electrogenic component reaction 2 is not influenced by the activation state of the ATPase. Consequently, it should be emphasized that the studies of Wise and Ort (1989) and Kramer and Crofts (1989), relating the decay of the overall P515 signal to the ATPase activation state might be in error. In these studies the contribution of the non-electrogenic component reaction 2, which is explicitly present in dark adapted intact plant material has not been taken into account.

The steady state flash-induced ATP synthesis can be related with the decay rate of reaction 1/RC (as shown in figure 4.6 and table 4.2). In a flash train of sixty 1 Hz flashes a steady state situation is reached, i.e. there is no further transmembrane charge accumulation upon following flashes. Consequently, under these conditions, we assume the electric field generated upon one flash to be dissipated before the following flash is fired. The ratio of k_1 and k_{atp} (table 4.2) reflects the ratio of charge dissipation through passive membrane leaks and through the proton conducting CF_0. The accelerated decay of reaction 1/RC under phosphorylating conditions is proposed to be due to proton conductance in the CF_0 part of the ATPase. Using approximate numbers of 1 electron transport chain (ETC) per 500 chlorophylls and a

CF_0/chlorophyll ratio 1/1000 (Ort 1986, Lill et al. 1986), a charge equivalent of 4 electrons is transported across the membrane upon each flash and per CF_0. With the values for k_1 and k_{atp}, 5.0 (s^{-1}) and 3.6 (s^{-1}), respectively (table 4.2), a proton flow of (3.6/8.6)*4 = 1.7 H^+ per CF_0 and per flash is calculated. Under these conditions the ATP yield is 0.6 nmol/mg Chl*flash (figure 4.2). Consequently, a H^+/ATP ratio of 2.8 is determined. This value is in good agreement with the general accepted H^+/ATP ratio of 3 (Davenport and McCarthy 1984, Junge 1987). This confirms the acceleration of the decay rate of the transmembrane electric field as sensed by reaction 1/RC to be stoichiometrically related to the ATP synthesis rate.

Additionally, using the value of k_{atp} the specific membrane conductance through the ATPase (g_{atp}) can be calculated. $g_{atp} = k_{atp}*c$, with a specific capacitance (c) of 1$\mu F/cm^2$ (Schoenknecht et al. 1986) a specific conductance of 3.6 $\mu S/cm^2$ is calculated. Using a surface density of chlorophyll molecules of 2.2 $(nm^2)^{-1}$ (Schoenknecht et al. 1986) a unit conductance of 0.1 fS is estimated. This value represents the conductance under coupled conditions and is, as expected, orders of magnitude lower than the values reported by others for CF_1-depleted CF_0 channels (Schoenknecht et al. 1986, Lill et al. 1986).

Conspicuous is the nearly fully inhibited decay of reaction 2 in the presence of ATP (figure 4.7 and table 4.3). This is in full agreement with earlier results (Schuurmans et al. 1981). Schreiber and Rienits (1982) showed an ATP induced dark absorbance change at 515 nm in chloroplast suspensions with an activated ATPase. This absorbance change coincides with a complementary loss of light induced absorbance change, and was attributed to the saturation of reaction 2 (Schreiber and Rienits 1982). Figure 4.7C illustrates the saturation of reaction 2 by ATP hydrolysis inducing a stationary higher absorbance level.

The presence of ATP and DTE can lead to autocatalytic activation of the ATPase (Davenport and McCarthy 1981). This activation is possibly intensified by the measuring beam. A slightly higher intensity of the measuring beam, indeed was found to lead to a lower amplitude of reaction 2 (not shown). This is presumably caused by an enhanced "zero" absorbance level resulting from partly saturation

of reaction 2 due to ATP hydrolysis in the dark. This will complicate the interpretation of the P515 signal in relation to ATPase activity. It needs no argument that signal averaging results in an uncontrolled saturation level of reaction 2 and should not be used in this type of experiments.

4.3.1 Reaction 2

It was earlier suggested that reaction 2 is related to innermembrane proton domains. This suggestion was based a.o. on the relative slow decay kinetics of reaction 2, its saturable character and the specific elimination of reaction 2 by low concentrations of CCCP (see figure 4.5) (Ooms et al. 1989, Peters et al. 1984). Based on different experimental approaches, other authors have suggested evidence for the existence of proton domains within the membrane (Theg and Junge 1983, Prochaska and Dilley 1978a, Prochaska and Dilley 1978b, Baker et al. 1981, Johnson et al. 1983, Beard and Dilley 1986). Both proton domains and reaction 2 show a similar sensitivity to ageing and temperature shock (Dilley et al. 1987). The saturation of reaction 2 by ATP hydrolysis (figure 4.7C) suggests the direct release of imported protons in the domain. This would be in agreement with the suggestion that protons released from photosystem 2 share a common sequestered region with the 8 kilodalton CF_0 subunit (Prochaska and Dilley 1978b, Baker et al. 1981). Moreover, in all our experiments a delayed onset of the ATP synthesis was allied to a smaller extent of reaction 2.

Here we want to speculate in more detail on the possible relation between reaction 2 and the loading and unloading of proton domains. According to the experiments of Beard and Dilley (1986), storage of chloroplasts in a high salt medium results in an increased number of flashes needed to initiate the ATP formation. Repeating those experiments, we found the same results. However, under the same experimental conditions, only small differences in the extent of reaction 2 were found. Based on the assumption that domains are open in the high salt stored chloroplasts (not accumulating protons), we expected considerable differences in the extent of reaction 2. The small difference might be explained by the finding of Allnutt et al. (1989);

they reported the proton domains in high salt stored chloroplasts to equilibrate with the lumen only after the initiation of electron transport, but before the energetic threshold for ATP synthesis is reached. Consequently, under these conditions, P515 absorbance changes upon the first flash cannot be expected to reveal substantial differences with regard to the status of proton domains.

In contrast we found a considerable difference in the onset flash lag and the extent of reaction 2 in assay medium A and B (figure 4.1 and 4.3), respectively. The energetic requirements for the onset of ATP synthesis, set by the redox state of the enzyme (Junesch and Gräber 1987), are unlikely to be different in both media. Following the suggested relation between innermembrane proton domains and reaction 2, it should be expected that in assay medium B (with a high onset flash lag and a decreased extent of reaction 2) the protons equilibrate with the thylakoid lumen, whereas in medium A, the protons do not, or at least much less. Beard and Dilley (1986) used the effect of the permeable buffer pyridine on the "onset flash lag" to determine whether proton domains are localized or delocalized. We adopted this method and found neither in medium A nor in medium B any effect of pyridine on the "onset flash lag" (not shown). Thus it is likely, in view of the interpretation of Dilley et al. (1987), that the proton domains are localized in both media. Furthermore, analysis of P515 signals indicate that the lower extent of reaction 2 in medium B is caused by an accelarated decay. The accelarated decay in medium B is supposed to be due to enhanced relaxation of a local electric field by others ions than protons from the domains. Taking into account the concurrent delayed onset of ATP synthesis in assay medium B, it is suggestive to conclude the localized electric field, to be essential in the onset of ATP synthesis.

Above we suggested no primary role for the electric potential in the onset of ATP synthesis. This was based on the finding that the transmembrane electric potential, sensed by reaction 1/RC, decays with a half time of about 110 ms, whereas the onset of ATP formation is frequency independent in the range of 0.2-5 Hz. In contrast, based on the finding of a delayed onset of ATP formation in the presence of valinomycin and potassium (Theg et al. 1988, Graan and Ort 1982), the transmembrane electric potential was reported to be crucial in the onset

of ATP synthesis. This seeming discrepancy might be based on the underestimation of an innermembrane localized electric field. In the presence of valinomycin, not only the transmembrane electric field, but also the local electric field associated with the proton domains and sensed as reaction 2 in the P515 signal, will be abolished (Schapendonk 1980). Consequently it can not be excluded that, under the conditions used, this local electric field is crucial in the onset of ATP synthesis, instead of the transmembrane electric field.

Possibly the different ionic conditions in assay medium B as compared to medium A, enhance the neutralization of the local electric field associated with proton accumulation. Assuming the local electric field to be crucial in the onset of ATP synthesis, we expect the "onset flash lag" to be frequency dependent under conditions where the decay of reaction 2 is accelerated (e.g. in assay medium B). In contrast, in assay medium B, the onset of ATP synthesis was found to be flash frequency independent (figure 4.2). This might be explained assuming the ΔpH generated upon the 15-18 flashes needed in medium B to initiate the ATP synthesis, to dominate the pmf needed for the onset of ATP synthesis in such a way that the local electric field sensed by reaction 2 becomes unimportant for the onset of ATP synthesis under these conditions.

4.3.2 Conclusions

Reaction 1/RC reflects the transmembrane electric field and its decay is stoichiometrically accelerated by the enhanced proton conductivity associated with ATP synthesis. Under ATP hydrolysing conditions the kinetics of reaction 1/RC are not altered. In interpreting the decay of the P515 signal as a measure of the activation state of the ATPase, one should take into account the contribution of the slow P515 component, reaction 2, which is very sensitive to light and ATP driven proton movements. Reaction 2 is presumably related to innermembrane proton accumulating domains. The decay kinetics of reaction 2 are not changed under conditions where the rate of ATP synthesis is altered.

5 REACTION 3, THE NON-ELECTROCHROMIC COMPONENT

5.1 Introduction

The flash-induced electrochromic bandshift measured at 518 nm consists of different components. Reaction 1/RC, reaction 1/Q and reaction 2 have been discussed in chapter 3 and 4. Here the fourth component, either called phase d (Schapendonk et al. 1979) or reaction 3 (Vredenberg et al. 1984), will be discussed. Reaction 3, which has to be corrected for when the P515 response is interpreted in terms of changes in electric field strength, is gramicidin insensitive. The amplitude is 5-10% of the maximum P515 absorbance change. It has slow decay kinetics, 2-5 seconds and a broad spectrum, different from P515 (Junge 1977, Vredenberg et al. 1984).

In this chapter the flash-induced gramicidin insensitive absorbance change, reaction 3, is considered in relation to the different oxidation reduction reaction involved in flash-induced electron tranport in thylakoid membranes.

5.2 Results

Figure 5.1 shows the spectrum of reaction 3, measured as the absorbance changes in the presence of gramicidin after 6 consecutive saturating flashes at a frequency of 5 Hz. In the wavelength range 500-600 nm small flash-induced absorbance changes of cytochrome f, cytochrome b_{563}, cytochrome b_{559}, plastocyanin, P700 and ferredoxin are known to contribute to the total absorbance change (de Wolf et al. 1988). However, the flash-induced absorbance changes of the above mentioned components, relax within 100-150 ms. This is shown in dual wavelength measurements (Jones and Whitmarsh 1985, Olsen et al. 1980) as well as in experiments where the overall absorbance changes are spectrally deconvoluted in the different components contributing to the signal

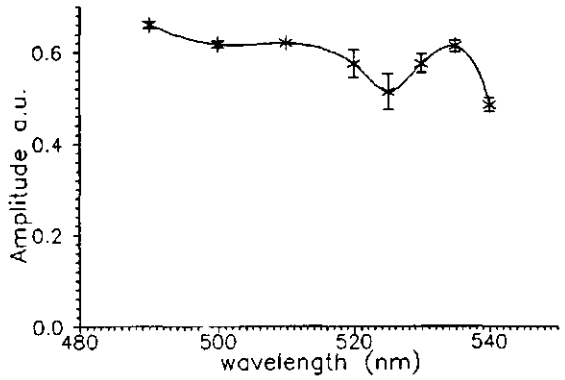

Figure 5.1 Spectrum of reaction 3 in broken spinach chloroplasts, defined as the absorbance change after 6 consecutive 5 Hz flashes in the presence of gramicidin. Spinach chloroplasts equivalent to 25 µg chlorophyll were broken in the cuvette prior to the experiment in reaction medium B: 330 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 75 mM HEPES/KOH pH 7.5. Further additions, 60 µM methylviologen and 5 µM gramicidin.

(de Wolf et al. 1988, Rich et al. 1987). At 490 nm contributions from the afore mentioned components are minimal. Moreover, the P515 signal at this wavelength is negligible (Garab et al. 1979). Consequently we have chosen 490 nm as the measuring wavelength for further studies of reaction 3.

The left column of figure 5.2 shows measurements of reaction 3 at 490 nm and the effects of the addition of DCMU, DCMU+DQH_2 and DCMU+DQH_2+DBMIB, respectively. The middle column shows the same experiments monitoring the effects of the different additions on cytochrome f. The right column shows the same experiments monitoring the redox state of P700. All measurements were carried out under the same conditions.

Figure 5.2A illustrates the accumulation of the reaction 3 absorbance change upon every flash and its slow decay kinetics after the flash train. Under the same conditions cytochrome f and P700 show a turnover on each flash. The spikes measured in the P700 signal are due to a flash artefact.

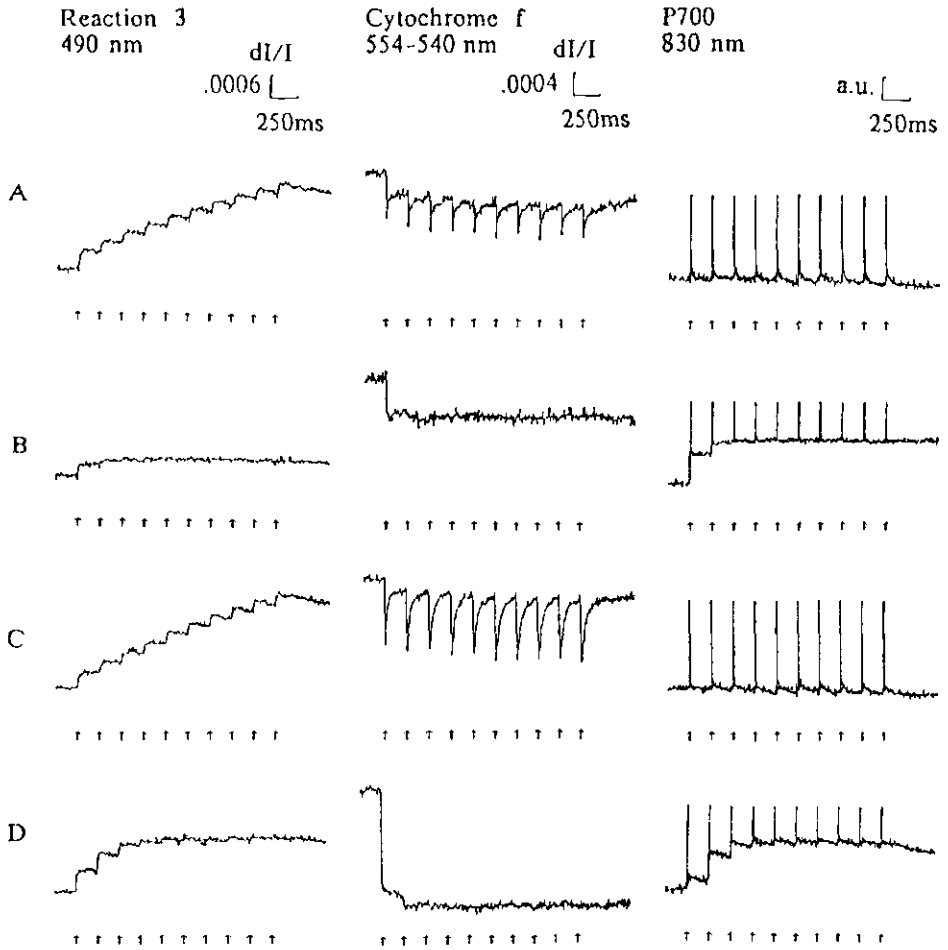

Figure 5.2. Flash-induced absorbance changes of reaction 3, cytochrome f and P700 of freshly broken spinach chloroplasts, in reaction medium B (see figure 5.1) in the presence of 60 µM MV and 5 µM gramicidin. A: control without further additions, B: with 10 µM DCMU, C: with 10 µM DCMU and 450 µM DQH_2, D: with 10 µM DCMU, 450 µM DQH_2 and 1.5 µM DBMIB. All signals are averages of 20 flash trains at 0.1 Hz.

The turnover of P700 is too fast to be monitored with our measuring equipment so only the redox state of steady state levels can be compared.

DCMU severely inhibits reaction 3, cytochrome f and P700 on the second flash, whereas on the third flash full inhibition is accomplished (fig 5.2B). In figure 5.2C reaction 3 and the turn-over of cytochrome f and P700 are fully restored by the addition of DQH_2. This suggests that the appearance of reaction 3 parallels the possibility to oxidize P700 (White et al. 1978). Figure 5.2D shows that this restoration can be inhibited by DBMIB. The second flash hardly oxidizes cytochrome f, while P700 is oxidized on the second flash. However, after the second flash P700 is only partly re-reduced, which causes P700 oxidation to be much lesser in the third flash. This correlates well with the lower amplitude of reaction 3 on the third flash. The correlation between the extent of reaction 3 and the redox state of P700, suggests a direct involvement of electron transport in or beyond photosystem 1.

Figure 5.3 shows a damped binary oscillation of reaction 3 in an experiment in which the electron acceptor MV is replaced by pBQ. Conspicuous, this oscillation, though strongly damped, was also found in the re-reduction of P700 measured under the same experimental conditions (figure 5.3). Qualitatively the same results were obtained with 5 mM ferricyanide as electronacceptor instead of pBQ. The experiments, illustrated in figure 5.2 and the concurrent binary oscillation of reaction 3 and P700 re-reduction, indicate the involvement of photosystem 1 in the induction of reaction 3.

Binary oscillations are generally interpreted to originate from the quinone reduction and oxidation reactions at the Q_B-site of photosystem 2 (Velthuys 1981, Laasch et al. 1983, Renger et al. 1988). Halogenated 1,4-benzoquinones have been reported to accept electrons from Q_A in a DCMU insensitive electron transfer reaction (Renger et al. 1987 and 1988). As shown in figure 5.2, DQH_2 restores reaction 3 in the presence of DCMU. We wanted to verify if DQH_2 under our conditions caused a bypass of the DCMU electron transport inhibition. To test this, we used the decay kinetics of the flash-induced fluorescence yield, as a measure for Q_A^- re-oxidation (Forbush and Kok 1968). However, we found no

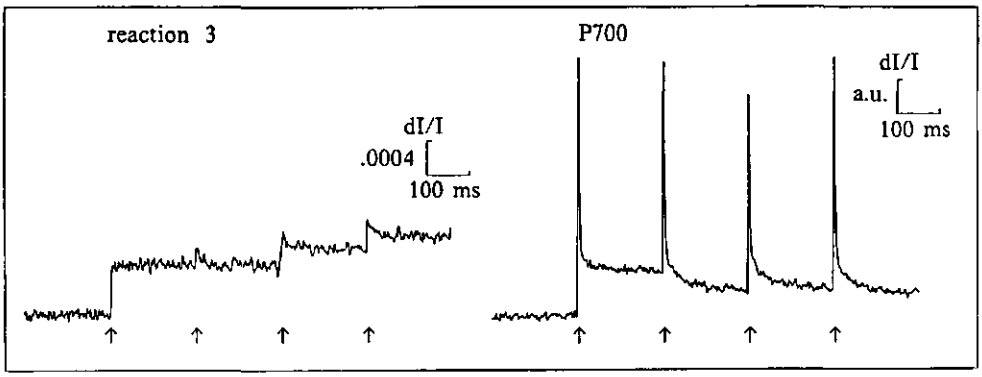

Figure 5.3. Flash-induced absorbance changes of reaction 3 and P700 of freshly broken spinach chloroplasts. Conditions as in fig 5.2A, except that 60 μM MV is replaced by 0.4 mM pBQ. Signals are averages of 20 flash trains at 0.1 Hz.

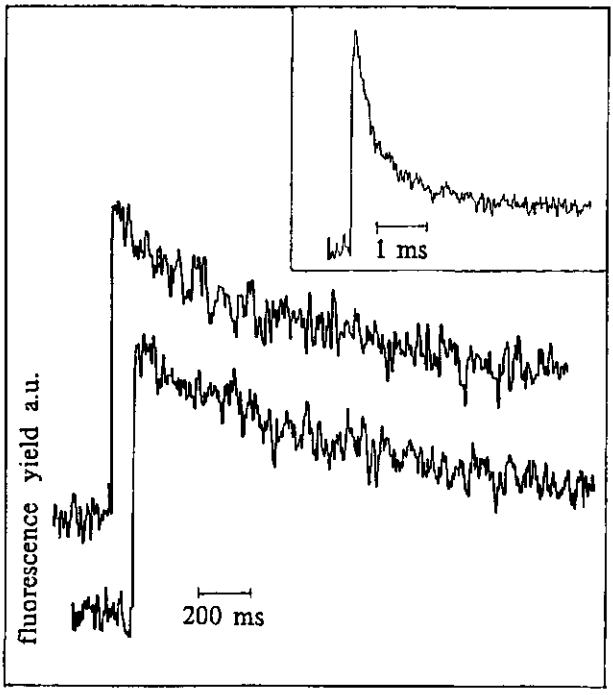

Figure 5.4. Flash-induced fluorescence changes in broken spinach chloroplasts. Measuring conditions as in figure 5.2A. Upper trace: with 0.05 μM DCMU, lower trace: with 0.05 μM DCMU and 7.5 μM DQH_2. Insert control experiment without additions. Note the different time scales. All traces are from single sweeps.

indication for a faster relaxation of the flash-induced fluorescence in the presence of DQH_2 (figure 5.4). Accordingly we concluded DQH_2 not to function as a bypass for DCMU electron transport inhibition.

In figure 5.5 pBQ was used as electronacceptor of photosystem 2 and DBMIB was used to block reduction of the cytochrome b/f complex at the plastoquinol oxidation site. In trace A reaction 3 is shown to be inhibited after 1-2 flashes. In trace C the P515 signal was monitored under the same experimental conditions, and reveals the amplitude of the initial fast rise, due to primary charge separation to be halved in

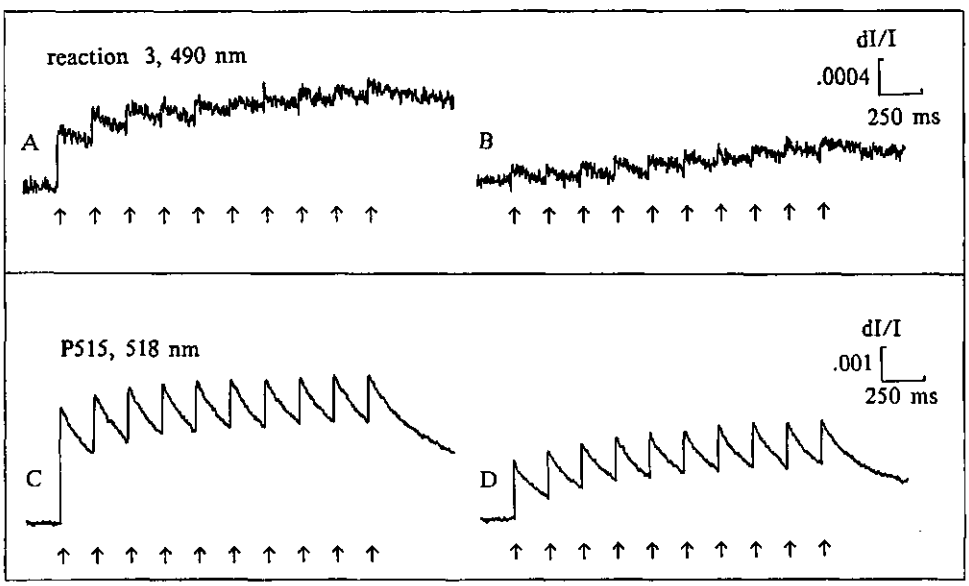

Figure 5.5. Flash-induced absorbance changes of reaction 3 and P515, in the absence (A, C) and presence (B,D) of far-red background illumination. Spinach chloroplasts were freshly broken in reaction medium B with addition of 0.4 mM pBQ and 1.5 μM DBMIB. Trace A and B: Reaction 3 monitored at 490 nm in the presence of 5 μM gramicidin, trace A: dark, trace B: with far-red background illumination. Trace C and D: P515 sinal, monitored at 518 nm in the absence of gramicidin, trace C: dark, trace D: with far-red background illumination. Signals are averages of 20 flash trains at 0.1 Hz.

in the second and following flashes. This is indicative for the inhibition of photosystem 1 electron transport by DBMIB. In the traces B and D, the same experiment is repeated, with a weak far-red background illumination, in order to oxidize photosystem 1 and its primary donors before the flash train starts. In the first flash of trace D, the amplitude of the initial rise of P515 is indeed halved compared to the control (trace C), indicating inhibition of photosystem 1 charge separation. Under the same experimental conditions the amplitude of reaction 3 upon the first and subsequent flashes is diminished (figure 5.5D). The turnover of P515 on every flash (trace C and D) is attributed to an uninhibited charge separation in photosystem 2. These experiments confirm the suggestion that reaction 3 is exclusively related to electron transport in or beyond photosystem 1.

5.3 Discussion

The spectrum of reaction 3 shown in figure 5.1, reveals a minimum around 525 nm, and shows a tendency to lower values around 540 nm. Although the absorbance values for the spectrum were taken 500 ms after the flashes, we suspect the absorbance change associated with the oxidation of cytochrome f to cause the dip in the spectrum. The difference spectrum (reduced minus oxidized) of cytochrome f shows a slight maximum around 525 nm (Bendall 1982). After its flash oxidation, cytochrome f is not fully re-reduced under control conditions (figure 5.2A). Accordingly, a small irreversible or very slow relaxing absorbance change due to cytochrome f oxidation contributes to the reaction 3 spectrum. This contribution is explicitly seen in the spectrum of reaction 3 around 525 nm. This interpretation is confirmed by measurements of the spectrum in the presence of DBMIB and DQH_2, respectively. DBMIB inhibits re-reduction of cytochrome f (see figure 5.2), and consequently was found to enhance the dip at 525 nm in the spectrum (not shown). In contrast DQH_2, stimulating re-reduction of cytochrome f abolishes the dip in the reaction 3 spectrum (not shown).

Binary oscillations in photosynthetic electron transport are associated with plastoquinone reduction and oxidation reactions (Velthuys 1981,

Petrouleas and Diner 1987). Renger et al. (1988) reported binary oscillations in the flash-induced absorbance change at 320 nm, the absorbance maximum of the difference spectra Q_A^-/Q_A and Q_B^-/Q_B, in the presence phenyl-p-benzoquinone. They found a higher initial amplitude on the first flash and a relative lower amplitude on the second flash. Although these findings compare well with the oscillation found in reaction 3 (figure 5.3) it seems unlikely to suppose that reaction 3 is associated with Q_A and/or Q_B absorbance changes for the following reasons: (i) At 490 nm the flash-induced absorbance changes of Q_A and Q_B are minimal (Schatz and van Gorkom 1985). (ii) The accumulation of absorbance change in reaction 3 upon a 5 Hz flash train is not found for Q_A or Q_B absorbance changes. (iii) DQH_2, restoring reaction 3 after DCMU inhibition had no effect on the relaxation of flash-induced Q_A fluorescence in the presence of DCMU (figure 5.4).

DAD/ascorbate is known to stimulate photosystem 1 associated cyclic electron tranport in a DBMIB insensitive way (Trebst 1980). We found DAD/ascorbate to restore the flash-induced reaction 3 absorbance change after inhibition of the signal by DCMU (not shown). Because of its DBMIB insensitivity, this result suggests no involvement of plastoquinol oxidation and consequently no involvement of an electrogenic Q-cycle in reaction 3. This is confirmed by our finding that HQNO, an inhibitor of the quinone reduction site (Q_r-site) of the cytochrome b/f complex, was unable to inhibit reaction 3 (not shown). This finding supports the suggestion that turnover of photosystem 1 is essential in the appearance of reaction 3.

Schreiber et al. (1988) reported binary oscillations in the extent of re-reduction of P700 under conditions when the plastoquinol pool was pre-oxidized with ferricyanide. Indeed with ferricyanide instead of pBQ as electronacceptor, we found qualitatively the same results as shown in figure 5.4. The binary oscillation in reaction 3 and P700 re-reduction is additional evidence for the suggestion that reaction 3 is directly associated with the oxidation of P700 or reduction of photosystem 1 primary acceptors. The binary oscillation in the P700 re-reduction was explained, presuming full oxidation of the PQ-pool by ferricyanide (Schreiber et al. 1988). Under these conditions the

immediate donorpool of photosystem 1 contains less electrons than are transported by P700 during a flash. Consequently the Q_B two electron gating mechanism governs the P700 re-reduction and possibly the extent of reaction 3.

From the results presented here, we conclude that reaction 3 is directly related to electron transport in photosystem 1. Due to its broad spectrum, revealing similarities with the spectrum of scattering changes under continuous illumination (Coughlan and Schreiber 1984a), reaction 3 has been suggested to be caused by scattering changes (Vredenberg et al. 1984). However, scattering changes during continuous illumination are related to membrane energization, and are strongly suppressed by ionophores or electron tranport inhibitors (Thorne et al.1975, Coughlan and Schreiber 1984a). Moreover, the slow light-induced 180° scattering (transmittance) changes during continuous illumination were proposed to be caused by large conformational changes like membrane flattening and appression of grana stacks (Coughlan and Schreiber 1984b). Accordingly, it seems inappropriate to presume flash-induced absorbance changes measured in the presence of gramicidin to be caused by large conformational changes related to membrane energization. However, slow (>500 ms) transients in flash-induced scattering have been attributed to alterations in the microenvironment and conformation of the particles responsible for scattering (Garab et al. 1979). It is suggestive to think that conformational changes related to the activation of ferredoxin-NADP reductase (Wagner et al. 1982) or the coupling factor complex (McCarthy 1979) cause slowly relaxing scattering changes.

Additionally it is clear that one should take care in interpreting small differences in the P515 signal. Small changes in the P515 signal might be due to not well characterized electron transport related non-electrochromic phenomena, like reaction 3.

6 GENERAL DISCUSSION

6.1 Introduction

The flash-induced P515 signal is generally recognized as a sensitive indicator for the transmembrane electric field across thylakoid membranes (Joliot and Delosme 1974, Witt 1979). Although it offers a proper tool in studies of photosynthetic electron transport and associated processes, some limitations were already earlier recognized. Absorbance changes at 518 nm associated with an alteration in the transmembrane electric field under continuous illumination are accompanied, and as such obscured, by scattering changes due to membrane energization (Thorne et al. 1975, Junge 1977, Coughlan and Schreiber 1984b).

Furthermore, flash-induced field indicating absorbance changes have to be corrected for a gramicidin insensitive non-electrochromic component either called phase d or reaction 3 (Junge 1977). In contrast to the recognition of the need to correct the flash-induced P515 signal for the non-electrochromic component reaction 3, the existence of a non-electrogenic component which we have called reaction 2 has insufficiently been recognized so far. In this chapter the impact of reaction 2 on common interpretations of the flash-induced P515 signal will be discussed.

6.2 The primary charge separation

The initial fast rise in the flash-induced P515 signal which occurs in the ps range (Leibl et al. 1989, Leibl and Trissl 1990) has been attributed to the primary charge separation in the reaction centers of photosystem 1 and 2. If reaction centers are closed, i.e. when their primary electron acceptors are in the reduced state, excitation of a reaction center will not lead to a transmembrane charge separation.

Thus, the extent of the initial fast absorbance change reflects the status of the reaction centers. This property is used a.o. to study the turnover rate of the photosystems and e.g. photosystem 2 heterogeneity (Chylla et al. 1987). It is shown (chapter 3) that both reaction 1/Q, associated with the operation of a transmembrane Q-cycle and the non-electrogenic component reaction 2 have a slow rising phase in the ms range and above. Thus in determining the extent of the fast initial rise there is no need to correct for the slow rising components reaction 1/Q and reaction 2.

With our measuring equipment we were not able to determine the rise kinetics of the non-electrogenic and non-electrochromic component reaction 3. Thus the initial fast rise as measured in our setup probably contains a contribution of the reaction 3 component. Consequently, if the extent of the fast rise is used to monitor the primary charge separation in the reaction centers, it needs to be corrected for the contribution of reaction 3.

6.3 The Q-cycle

Since its recognition (Joliot and Delosme 1974) there has been ample debate on the origin of the slow rising phase in the P515 signal. Charge delocalization in the appressed membrane region (Olsen and Barber 1981, Zimányi and Garab 1982), cyclic electron transport around photosystem 1 (Crowther and Hind 1980, Shahak et al. 1980), additional charge separation due to a functional Q-cycle or modifications thereof (a.o. Jones and Whitmarsh 1988) and localized innermembrane proton domains (Schapendonk 1979, Van Kooten et al. 1983) have been suggested as explanations for the slow rise in the P515 signal.

The main confusion in studies and discussions on the "slow phase" in the P515 signal is caused by the fact that it has insufficiently been recognized, if at all, to be composed of two distinguishable processes, which both give rise to an electrochromic absorbance change. As shown in chapter 3, the "slow phase" is a composite of the electrogenic reaction 1/Q and the non-electrogenic reaction 2. In literature the "slow phase" is commonly assumed to reflect a transmembrane electrogenic charge separation associated with a

functional Q-cycle. This interpretation, however, is correct only for experimental conditions when reaction 2 is fully suppressed or saturated. Under these conditions reaction 1/Q determines the slow rising phase.

Reaction 2 has been shown to be sensitive, amongst others, to a temperature shock, ageing and membrane modifying agents like valinomycin and CCCP. Moreover, its extent has been reported to depend strongly on the mildness of the chloroplast isolation procedure (Peters 1986). Reaction 2 can be partly saturated by a weak pre- or background illumination. Saturation of reaction 2 is particularly stimulated under conditions where illumination activates ATP hydrolysis (chapter 4). Consequently the extent of reaction 2 in the flash-induced P515 signal will be much smaller under these conditions. As shown in chapter 3, the presence of reaction 2 in the P515 signal has great impact on the overall kinetics of the signal. The half decay time of reaction 1/RC and reaction 1/Q is about 100 ms (chapter 3), whereas the decay of reaction 2 is much slower (halftime 500-1000 ms). Accordingly it seems easy to determine whether reaction 2 contributes to the overall flash-induced P515 signal.

There are arguments to presume that under the experimental conditions used in the work of other authors (Bouges Bocquet 1981, Selak and Whitmarsh 1982, Moss and Bendall 1984, Jones and Whitmarsh 1985, Barabás et al. 1985a and 1985b, Moss and Rich 1987, Rich 1988, Jones and Whitmarsh 1988, Rich 1990), studying and using P515 as a potential probe of the electrogenic Q-cycle, reaction 2 has been saturated or suppressed. For example, Rich (1990) used chloroplasts stored in liquid nitrogen, and reported a full decay of the overall P515 signal in about 200 ms. Jones and Whitmarsh (1985), working with naked lamellae, found a half time for the decay of the P515 signal of 75 ms for both, the fast and the slow rising phase. Pre-treatment of samples with approximately 10 flashes as performed by Jones and Whitmarsh (1988) will surely lead to a partial saturation of reaction 2. Signal averaging with relative high repetition rates (0.1-0.2 Hz), generally applied to enhance the signal to noise ratio will also lead to an underestimation of the extent of reaction 2 due to its (partial) saturation.

However the slow decay of the overall flash-induced P515

signal (about 3 sec) measured in intact algae (Joliot and Joliot 1986b) and (about 830 ms) in chloroplasts (Hope and Matthews 1987) suggests that under the conditions used by these authors a reaction 2 component is present. These authors, (Joliot and Joliot 1986b and Hope and Matthews 1987) reported a smaller slow phase under repetitive flash experiments compared to single flash experiments. This is in confirmation with the experiments of Garab et al. (1987) and our interpretation of a (partial) saturation of reaction 2 under repetitive flash experiments (chapter 3 and 4).

A serious challenge for a functional Q-cycle explaining the slow electrogenic phase in the P515 signal has been the finding that cytochrome b_6 turnover is not obligatorily allied to the "slow phase" in the P515 signal (Girvin and Cramer 1984, Moss and Rich 1987). Moreover, in Chlorella cells the oxidation of pre-reduced cytochrome b and the generation of a slow electrogenic P515 response were not inhibited by the Q_r-site inhibitor NQNO (Joliot and Joliot 1986b). These findings suggested a non obligate association between the "slow rising component" and the function of a transmenbrane Q-cycle. Girvin and Cramer (1984) suggested a transmembrane proton pump coupled to the reduction of Rieske iron-sulfur center as an explanation for a "slow P515 response" which is not associated with cytochrome b turnover. Joliot and Joliot (1986a and 1986b) suggested the possibility of reduced cytochrome b to be oxidized by a semiquinone at the Q_o-site. This oxidation was presumed to be coupled to a transmembrane proton pump which generates the "slow P515 response". Although the rise kinetics of reaction 1/Q and reaction 2 are different (see table 3.1), it cannot be excluded at forehand that the slow rising phase detected in the above mentioned experiments was partly contaminated with a contribution of reaction 2. Consequently, the alternatives for the Q-cycle explaining the "slow phase" in the P515 signal (Girvin and Cramer 1984, Joliot and Joliot 1986b) might be based on a serious underestimation of the reaction 2 component in the slow phase of P515 under the conditions used.

In freshly isolated intact chloroplasts it has convincingly been shown that reaction 2 dominates the overall P515 signal (see figure 3.6 and 3.7 for illustration). It is not surprisingly that the relative small component,

reaction 1/Q, which originates from an electrogenic Q-cycle was "hidden" and has been overlooked under these conditions (Schapendonk 1979, Peters et al. 1984, 1986, Van Kooten 1983). Thus from their research on "the slow phase", exclusively referred to as reaction 2, it was concluded that the slow response did not originate from an electrogenic Q-cycle. This was amongst others based on, and discussed in terms of the decay kinetics of reaction 2, being too slow to reflect an electrogenic process (Vredenberg 1981).

From the results presented in chapter 3, it is clear that two kinetically different slow components can be distinguished in the flash-induced P515 signal. Consequently, the generally used terminology "the slow phase" is insufficiently precise and incomplete to describe the complex kinetics of the flash-induced P515 response. To avoid further confusion, it needs to be specified whether reaction 1/Q, reaction 2 or both reactions are monitored.

6.4 The decay kinetics and ATPase activity

Besides the application of the P515 signal to monitor the operation of a functional Q-cycle in chloroplasts and intact cells, the decay rate of the overall P515 signal is used as an indicator of the thylakoid membrane ATPase activation state. The accelerated decay of the P515 signal under phosphorylating conditions is commonly associated with ATP synthesis coupled proton conductivity through the ATPase (Junge et al. 1970, Witt 1979, Girault and Galmiche 1978, Morita et al. 1982, Raines and Hipkins 1988, Kramer and Crofts 1989, Wise and Ort 1989, Bogdanoff and Gräber 1990).

In chapter 3 it is illustrated that reaction 2 dominates the overall P515 signal. As documented in detail elsewhere (Peters 1986) and in chapter 4, reaction 2 can be saturated either by a few shortly spaced flashes or by ATP hydrolysis. Under these conditions, the flash-induced P515 signal is enriched in the reaction 1/RC component, giving rise to an apparent accelerated decay (fig 4.7). As mentioned before, reaction 2 is not, or insufficiently recognized in most experimental results. Consequently, the strong acceleration of the overall decay of the P515 signal under phosphorylating conditions or after a few shortly

spaced actinic flashes, might be erroneously taken as evidence for an enhanced proton conductivity through the ATPase.

It should be stressed that the saturation of reaction 2 can be caused by ATP hydrolysis. Thus, the apparent acceleration of the overall P515 signal might indicate ATP hydrolysis. However, as shown by Peters et al. (1985), even under conditions where ATP hydrolysis is 95% inhibited by DCCD, reaction 2 is still suppressed. Accordingly, a strong apparent acceleration of the overall P515 signal, indicative for the saturation of reaction 2, is surely not a quantitative measure for ATPase activity.

Kramer and Crofts (1990) described a newly developed instrument for measurements of flash-induced absorbance changes in intact leaves. Instead of the conventional low intensity measuring beam, a series of relative high intensity measuring pulses were used to increase the signal to noise ratio. The advantage of this method is that no averaging is needed.

Although the integrated intensity of the measuring pulses over the course of the experiment remains low and non-actinic (Kramer and Crofts 1990), it cannot be excluded that the measuring pulse itself has actinic effects. Due to the differential measurement of two closely spaced leaf areas, the possible actinic effect of the measuring pulse, inducing P515 absorbance changes, is fully neglected. This might lead to misinterpretations of the flash-induced P515 signal.

Nevertheless, using this newly developed instrument, some elegant measurements of the flash-induced absorbance changes in intact cucumber leaves have been carried out (Kramer and Crofts 1989). The acceleration of the overall flash-induced P515 signal after light adaptation was found to be inhibited by the external addition of DCCD. These experiments can be exposed to the same criticism as those of Morita et al. (1982) (Peters et al. 1985). Kramer and Crofts (1989) maintained a two minutes dark period before the flash-induced absorbance change was measured. Peters et al. (1985) showed the dark recovery of the slow decaying phase reaction 2 in the presence of DCCD in isolated chloroplasts to be 2 minutes, whereas the dark recovery in the control experiment took 30 minutes. Thus, the experiments of Kramer and Crofts (1989) might suggest the saturation

of reaction 2 (erroneously interpreted as acceleration of the overall P515 signal) to be inhibited by DCCD. In fact, DCCD prevents prolonged ATP hydrolysis to maintain the saturation of reaction 2 during a longer time interval after the light adaptation.

In chapter 4 in parallel experiments the ATP synthesis rate and the decay of the flash-induced P515 signal were monitored. In confirmation with Ooms et al. (1990a) it is shown that the single exponential decay of the transmembrane electric field monitored by reaction 1/RC is stoichiometrically related with the ATP synthesis rate. In contrast, the decay rate of the reaction 2 component is not influenced by the ATP synthesis rate. Reaction 2 can be saturated either by a few pre-illuminating flashes or by ATP hydrolysis. This saturable character of reaction 2 and its sensitivity to light and ATP driven proton movements should be taken into account in experiments where the decay of the P515 signal is related to the ATP synthesis rate. Consequently an accurate deconvolution of the P515 signal in its different components is needed to relate P515 signals to ATP synthesis rates.

6.5 Reaction 2

Most controversial discussions and interpretations concerning the flash-induced P515 signal have their origin in different interpretations of the slow rising phase. The recognition of two distinguishable slow rising phases, reaction 1/Q and the non-electrogenic phase reaction 2, will contribute to the clarification of these controversies.

Reaction 2 has been suggested to originate from innermembrane proton domains (Schapendonk 1980, Vredenberg 1981, Van Kooten 1988). This suggestion was based on (i) the relative slow decay kinetics of reaction 2, which are unlikely to be associated with electrogenic phenomena, (ii) the saturable character of reaction 2 and (iii) its specific elimination by low concentrations of membrane modifying agents like valinomycin and CCCP. Other authors (Theg and Junge 1983, Sigalat et al. 1985, Dilley et al. 1987) using different experimental approaches have also suggested the existence of innermembrane proton domains which are not in equilibrium with the

bulk aqueous phases.

The results presented in chapter 4 provide further evidence for the still speculative suggestion that reaction 2 reflects the generation of an innermembrane electric field associated with proton accumulation.

If our concept is correct, the decay of reaction 2 might be determined either by proton release from the proposed domains or by electric compensation of the localized charge by other ions. The results presented in chapter 4 revealed that an enhanced number of flashes needed for the onset of ATP synthesis in assay medium B is accompanied by a smaller extent of reaction 2 under the same experimental conditions. The number of flashes needed for the onset of ATP synthesis has been suggested to be a qualitative measure of the status (localized or delocalized) of proton domains associated with the ATPase (Beard and Dilley 1986, Dilley et al. 1987). Thus, it is tentative to suggest this decrease of reaction 2 to be due to the delocalized state of the proton domains. However, it was shown that under the different conditions used (assay medium A and B) the proton domains do not equilibrate with the thylakoid lumen. This suggests that the decay of reaction 2 is determined by the displacement of other ions than protons, neutralizing the local electric field.

Other authors (Theg and Junge 1983, Laszlo et al. 1984b) reported prolonged periods during which the thylakoid membranes can retain protons in the dark. Consequently, under their conditions it is also inappropriate to propose the equilibration of domain protons with the thylakoid lumen to be an important factor determining the decay rate of reaction 2.

Nevertheless it cannot be excluded that under experimental conditions with the proton domains in a delocalized state (e.g. using high salt stored chloroplasts, (Beard and Dilley 1986) or in the presence of low concentrations of the proton ionophore CCCP), the release of protons from the domains does significantly contribute to the decay of reaction 2.

Although the study presented in this thesis provides additional support for the suggestion that the reaction 2 component of the flash-induced P515 signal is associated with the release and stabilization of protons in innermembrane domains, the definite origin of reaction 2 still remains to be elucidated.

Summary

This thesis deals mainly with the analysis and interpretation of the flash-induced electrochromic absorbance changes in isolated chloroplasts of spinach and pea plants. The amplitude and kinetics of the flash-induced absorbance changes at 518 nm (P515) are discussed in relation to the functioning of the photosynthetic electron transport and energy coupling mechanisms, which are associated with the thylakoid membrane.

At least four different components can be distinguished in the flash-induced P515 signal in intact chloroplasts. Reaction 1/RC reflects the primary charge separation in the photosynthetic reaction centers. It shows a fast rise and single exponential decay kinetics. It is shown that the generally recognized "slow rising phase" in fact is composed of at least two distinguishable components related to different membrane processes. One component called reaction 1/Q is associated with the functioning of an electrogenic Q-cycle, whereas the other slow component, called reaction 2, is non-electrogenic, and is proposed to be associated with innermembrane proton domains. The two slow components can be discriminated based on their different rise and decay kinetics and the different stimulatory and inhibitory effects of DQH_2, DBMIB and CCCP, respectively.

A fourth component in the flash-induced P515 signal is called reaction 3. This component which contributes only 5-10% to the overall P515 signal is non-electrochromic and non-electrogenic. With the aid of different electron transport inhibitors and electron-donors the origin of reaction 3 is located more precisely. Under certain experimental conditions, reaction 3 exhibits a binary oscillation. Although this oscillation most probably originates from the Q_B two electron gating mechanism at the reducing site of photosystem 2, it is shown that the occurence of reaction 3 in the flash-induced absorbance change is directly associated with the electron transport in photosystem 1.

The kinetics of the distinguishable P515 components are studied in relation to the onset of ATP synthesis and the steady state ATP synthesis rate. The dissipation of the transmembrane electric field as monitored by reaction 1/RC is stoichiometrically related to the ATP synthesis rate. The decay of the non-electrogenic saturable component, reaction 2, is not influenced by the ATP synthesis rate. Consequently it is concluded that the decay of the overall P515 signal cannot be used as a proper measure of the ATP synthesis rate.

Under the experimental conditions used, an enhanced number of flashes necessary for initiating ATP synthesis is accompanied by a lower extent of reaction 2. This finding is discussed within the frame of the earlier suggested relation between reaction 2 and the existence of innermembrane proton domains.

Finally, some aspects of the use of the flash-induced P515 signal as a tool in studies of the photosynthetic electron transport and energy coupling are discussed. The P515 signal is used to monitor e.g. i) the extent of the primary charge separation, which is related to the redox state of the primary electron acceptors, ii) the functioning of an electrogenic Q-cycle and iii) the activation state of the thylakoid membranes ATPase.

It is emphasized that the non-electrogenic saturable character of the reaction 2 component which is sometimes insufficiently recognized in experimental work of others, is a possible source of misinterpretations and confusion concerning the kinetics of the P515 signal.

Samenvatting

In fotosynthetische membranen (thylakoidmembranen) vindt onder invloed van licht een ladingsscheiding plaats. Het elektrische veld dat deze ladingsscheiding veroorzaakt heeft een kleine absorptie verandering tot gevolg in het zogenaamde P515 pigment komplex. Deze absorptie verandering vormt een lineaire maat voor het transmembraan elektrisch veld, en kan dus gebruikt worden om het ontstaan en het verval van dit elektrische veld te bestuderen.

In dit proefschrift wordt de flitsgeïnduceerde absorptie verandering zoals die gemeten kan worden in geïsoleerde chloroplasten van spinazie- en erwtenplanten geanalyseerd en geïnterpreteerd. De amplitude en de kinetiek van de gemeten signalen wordt besproken in relatie tot het fotosynthetische elektronentransport en de energiekoppeling in de thylakoidmembranen.

In het flitsgeïnduceerde P515 signaal in intakte chloroplasten kunnen minstens vier verschillende komponenten onderscheiden worden. Reaktie 1/RC wordt veroorzaakt door de primaire ladingsscheiding in de fotosynthetische reaktiecentra. Deze reaktie vertoont een zeer korte stijgtijd en een enkelvoudig exponentieel verval. Naast deze snel stijgende reaktie wordt in het algemeen ook een trage komponent onderscheiden. In hoofdstuk 3 wordt aangetoond dat deze zogenaamde trage komponent in feite bestaat uit twee verschillende komponenten die gerelateerd kunnen worden aan twee verschillende processen. De eerste komponent die hier reaktie 1/Q wordt genoemd, wordt veroorzaakt door secundair elektrogeen ladingstransport in een funktionele Q-cyclus. De andere trage komponent die reaktie 2 genoemd wordt, is niet elektrogeen en staat waarschijnlijk in verband met membraangebonden protondomeinen. Het onderscheid tussen deze twee trage komponenten wordt gemaakt op grond van hun verschillende stijg- en vervaltijden en de verschillende stimulerende en remmende effekten van o.a. DQH_2, DBMIB en CCCP.

Een vierde komponent in het flitsgeïnduceerde P515 signaal is

reaktie 3. Reaktie 3 draagt slechts 5-10% bij aan het totale P515 signaal en is noch elektrogeen noch elektrochroom. Met behulp van verschillende remmers en elektrondonoren voor het fotosynthetische elektrontransport is de oorsprong van reaktie 3 nader gelokaliseerd. Onder bepaalde experimentele kondities vertoont de amplitude van reaktie 3 een binaire oscillatie die waarschijnlijk samenhangt met de binaire oscillatie die ook gevonden wordt in de Q_B reduktie in fotosysteem 2. Desalniettemin wordt hier aangetoond dat de bijdrage van reaktie 3 aan de flitsgeïnduceerde absorptie verandering direkt gerelateerd is aan het elektronentransport in fotosysteem 1.

De kinetiek van de verschillende komponenten in het P515 signaal is verder bestudeerd in relatie tot de start van de flitsgeïnduceerde ATP-produktie en de steady state ATP-produktie. Het verval van het transmembraan elektrische veld (reaktie 1/RC) is kwantitatief gerelateerd aan de snelheid van ATP-produktie. Het verval van de niet elektrogene verzadigbare komponent reaktie 2 wordt echter op geen enkele wijze beïnvloed door snelheid van ATP-produktie. Daaruit is geconcludeerd dat het verval van het totale P515 signaal niet geschikt is om de ATP-produktiesnelheid te meten of de aktiveringstoestand te bepalen. Onder bepaalde experimentele kondities bleek dat een toename van het aantal flitsen dat nodig is om de ATP-produktie op gang te brengen vergezeld gaat van een kleinere bijdrage van reaktie 2 in het P515 signaal. Dit effekt wordt nader besproken in het kader van de eerder gesuggereerde relatie tussen reaktie 2 en membraangebonden protondomeinen.

Tot slot worden een aantal aspekten van het gebruik van het flitsgeïnduceerde P515 signaal als instrument in studies van fotosynthetische elektronentransport en energiekoppeling, besproken. Het P515 signaal wordt onder andere gebruikt om de grootte van de primaire ladingsscheiding te bepalen, als indikator voor het funktioneren van een Q-cyclus en als maat voor de aktiveringstoestand van het ATPase. Met nadruk moet gesteld worden dat de verzadigbare niet-elektrogene komponent, reaktie 2, in veel experimenteel werk niet of onvoldoende onderkend wordt. Dit is mogelijk de oorzaak van de vele verwarrende discussies met betrekking tot de interpretatie van het flitsgeïnduceerde P515 signaal.

References

Albertsson PA (1985) Subfractionation of inside-out thylakoid vesicles and the localization of cytochrome f. Physiol Vég 25: 731-739.

Allnutt FCT, Dilley RA and Kelly T (1989) Effect of high KCl concentrations on membrane-localized metastable proton buffering domains in thylakoids. Photosynth Res 20: 161-172.

Anderson JM and Andersson B (1982) The architecture of photosynthetic membranes: lateral and transverse organization. Trends in Biochem Sci 7: 288-292.

Anderson JM and Andersson B (1988) The dynamic photosynthetic membrane and regulation of solar energy conversion. Trends in Biochem Sci 13: 351-355.

Andersson B and Åkerlund HE (1987) Proteins of the oxygen evolving complex. In: The light reactions. (Barber J ed) Elsevier Amsterdam New York Oxford.

Andréasson LE and Vänngård T (1988) Electron transport in photosystems 1 and 2. Ann Rev Plant Physiol 39: 379-411.

Arntzen CJ (1972) Inhibition of photophosphorylation by tentoxin, a cyclic tetrapeptide. Biochim Biophys Acta 283: 539-542.

Baker GM, Bhatnagar D and Dilley RA (1981) Proton release in photosynthetic water oxidation: evidence for proton movement in a restricted domain. Biochem 20: 2307-2315.

Barabás K, Zimányi L and Garab G (1985a) Kinetics of the flash-induced electrochromic absorbance change in the presence of background illumination. Turnover rate of the electron transport. I. Isolated intact chloroplasts. J Bioenerg Biomembr 17: 349-364.

Barabás K, Zimányi L and Garab G (1985b) Kinetics of the flash-induced electrochromic absorbance change in the presence of background illumination. Turnover rate of the electron transport. II. Higher plant leaves. J Bioenerg Biomembr 17: 365-373.

Beard WA and Dilley RA (1986) A shift in chloroplast energy coupling by KCl from localized to bulk phase delocalized proton gradients. FEBS-Lett 201: 57-62.

Beard WA and Dilley RA (1988a) ATP formation onset lag and post-illumination phosphorylation initiated with single-turnover flashes. I An assay using luciferin-luciferase luminescence. J Bioenerg Biomemb 20: 85-106.

Beard WA, Chiang G and Dilley RA (1988) ATP formation onset lag and post-illumination phosphorylation initiated with single-turnover flashes. II Two modes of post-illumination phosphorylation driven by either delocalized or localized proton gradient coupling. J Bioenerg Biomembr 20: 107-128.

Beard WA and Dilley RA (1988b) ATP formation onset lag and post-illumination phosphorylation initiated with single-turnover flashes. III Characterization of the ATP formation onset lag and post-illumination phosphorylation for thylakoids exhibiting localized or bulk-phase delocalized energy coupling. J Bioenerg Biomembr 20: 129-154.

Bendall DS (1982) Photosynthetic cytochromes of oxygenic organisms. Biochim Biophys Acta 683: 119-151.

Bogdanoff P and Gräber P (1990) Proton efflux and phosphorylation in flash groups. In: Current Research in Photosynthesis (Baltscheffsky M ed) Vol III pp. 217-220. Kluwer Academic Publishers, Dordrecht, Boston, London.

Borchard A and Junge W (1990) The effect of pyridine on the onset lag of photophosphorylation: no evidence for localized coupling. In: Current research in photosynthesis (Baltscheffsky M ed) vol III pp. 97-100. Kluwer Academic Publishers, Dordrecht, Boston, London.

Bouges-Bocquet B (1981) Factors regulating the slow electrogenic phase in green algae and higher plants. Biochim. Biophys. Acta 635: 327-340.

Bruinsma J (1963) The quantitative analysis of chlorophylls a and b in plant extracts. Photochem Photobiol 2: 241-249.

Carrillo N and Vallejos H (1987) Ferredoxin-$NADP^+$ oxidoreductase In: The light reactions. (Barber J ed) Elsevier Amsterdam New York Oxford.

Chylla RA ,Garab G and Whitmarsh J (1987) Evidence for slow turnover in a fraction of photosystem 2 complexes in thylakoid membranes. Biochim Biophys Acta 894: 562-571.

Coughlan SJ and Schreiber U (1984a) Light-induced changes in the conformation of spinach thylakoid membranes as monitored by 90° and 180° scattering changes: A comparative investigation. Z Naturforsch 39c: 1120-1127.

Coughlan SJ and Schreiber U (1984b) The differential effects of short-time glutaraldehyde treatments on the light-induced thylakoid membrane conformational changes, proton pumping and electron transport properties. Biochim Biophys Acta 767: 606-617.

Cramer WA, Black MT, Widger WR and Girvin ME (1987) Structure and function of photosynthetic cytochrome b-c_1 and b_6-f complexes. In: The light reactions. (Barber J ed) Elsevier Amsterdam New York Oxford.

Crielaard W, Cotton NPJ, Jackson JB, Hellingwerf KJ and Konings WN (1988) The transmembrane electrical potential in bacteria: simultaneous measurements of carotenoid absorbance changes and lipophilic cation distribution in intact cells of Rhodobacter sphaeroides. Biochim Biophys Acta 932: 17-25.

Crowther D and Hind G (1980) Partial characterization of cyclic electron transport in intact chloroplasts. Arch Biochem Biophys 204: 568-577.

Davenport JW and McCarthy R (1984) An analysis of proton fluxes coupled to electron transport and ATP synthesis in chloroplast thylakoids. Biochim Biophys Acta 766: 363-374.

Davenport JW and McCarthy RE (1981) Autocatalytic activation of the thylakoid ATPase. in Photosynthesis (Akoyunoglou G ed) Vol II pp. 859-865, Balaban Intern. Sci. Serv., Philadelphia.

De Wolf FA, Krab K, Vischers RW, de Waard JH and Kraayenhof R (1988) Characteristics and reinterpretation of single-turnover cyclic electron transfer. Biochim Biophys Acta 936: 487-503.

Dilley RA and Schreiber U (1984) Correlation between membrane-localized protons and flash-driven ATP formation in chloroplast thylakoids. J Bioenerg Biomembr 16: 173-193.

Dilley RA (1986) Proton-Membrane interactions in chloroplast thylakoids. In: Encyclopedia of Plant Physiology New Series Vol 19. Photosynthesis III, Photosynthetic membranes and light harvesting systems. (Staehelin LA and Arntzen CJ eds) Springer Verlag, Berlin Heidelberg.

Dilley RA, Theg SM and Beard WA (1987) Membrane-proton interactions in chloroplast bioenergetics: localized proton domains. Ann Rev Plant Physiol 38: 347-389.

Ferguson SJ (1985) Fully delocalized chemiosmotic or localized proton flow pathways in energy coupling? A scrutiny of experimental evidence. Biochim Biophys Acta 811: 47-95.

Forbush B and Kok B (1968) Reaction between primary and secondary electron acceptors in photosystem 2 of photosynthesis. Biochim Biophys Acta 162: 243-253.

Fork DC and Satoh K (1986) The control by state transitions of the distribution of excitation energy in photosynthesis. Ann Rev Plant Physiol 37: 335-361.

Garab GI, Paillotin G and Joliot P (1979) Flash-induced scattering transients in the 10 μs-5 s time range between 450 and 540 nm with *chlorella* cells. Biochim Biophys Acta 545: 445-453.

Garab G, Farineau J and Hervo G (1987) Dependence of energization of thylakoids on frequency of exiting flashes in intact chloroplasts. Photosynth Res 11: 15-27.

Girault G and Galmiche JM (1978) Effects of nucleotides on potential and pH changes across the thylakoid membranes of spinach chloroplasts Biochim Biophys Acta 502: 430-444.

Girvin ME and Cramer WA (1984) A redox study of the electron transport pathway responsible for generation of the slow electrochromic phase in chloroplasts. Biochim Biophys Acta 767: 29-38.

Graan T and Ort DR (1982) Photophosphorylation associated with synchronous turnovers of the electron-transport carriers in chloroplasts. Biochim Biophys Acta 682: 395-403.

Haehnel W (1984) Photosynthetic electron transport in higher plants. Ann Rev Plant Physiol 35: 659-693.

Hangarter R and Ort DR (1985) Cooperation among electron-transfer complexes in ATP synthesis in chloroplasts. Eur J Biochem 149: 503-510.

Hangarter R and Ort DR (1986) The relation between light-induced increase in the H^+-conductivity of thylakoid membranes and activity of the coupling factor. Eur J Biochem 158: 7-12.

Hangarter R, Jones RW, Ort DR and Whitmarsh J (1987) Stoichiometries and energetics of proton translocation coupled to electron transport in chloroplasts Biochim Biophys Acta 890: 106-115.

Haraux F (1986) Integrated functioning of the chloroplast coupling factor. Biochimie 68: 435-449.

Heber U and Santarius KA (1970) Direct and indirect transfer of ATP and ADP across the chloroplast envelope. Z Naturforsch 25B: 718-728.

Hope AB and Matthews DB (1987) The slow phase of the electrochromic shift in relation to the Q-cycle in thylakoids. Aust J Plant Physiol 14: 29-46.

Hope AB and Rich PR (1989) Proton uptake by the cytochrome bf complex Biochim Biophys Acta 975: 96-103.

Jahns P and Junge W (1989) The protonic shortcircuit by DCCD in photosystem 2. A common feature of all redox transitions of water oxidation. FEBS-lett. 253: 33-37.

Janowitz A, Günther G and Laasch H (1990) Localized proton domains in pH-dependent control of photosynthetic electron transport under the influence of lipophilic tertiary amines. In: Current research in photosynthesis. (Baltscheffsky M ed) vol IV pp. 211-215. Kluwer Academic Publishers, Dordrecht, Boston, London.

Johnson JD, Pfister VR and Homann PH (1983) Metastable proton pools in thylakoids and their importance for the stability of photosystem 2. Biochim Biophys Acta 723: 256-265.

Joliot P and Delosme R (1974) Flash-induced 519nm absorbance changes in green algae. Biochim Biophys Acta 357: 267-284.

Joliot P and Joliot A (1986a) Mechanisms of proton-pumping in the cytochrome b/f complex. Photosynth Res 9: 113-124.

Joliot P and Joliot A (1986b) Proton pumping and electron transfer in the cytochrome b/f complex of algae. Biochim Biophys Acta 849: 211-222.

Jones RW and Whitmarsh J (1985) Origin of the electrogenic reaction in the chloroplast cytochrome b/f complex. Photobiochem Photobiophys 9: 119-127.

Jones RW and Whitmarsh J (1987) The electrogenic reaction and proton release during quinol oxidation by the cytochrome b/f complex. In: Biggens J (ed) Progress in Photosynthesis Research Vol 2 pp. 445-452. Martinus Nijhoff Publishers, Dordrecht.

Jones RW and Whitmarsh J (1988) Inhibition of electron transfer and the electrogenic reaction in the cytochrome b/f complex by 2-n-nonyl-4-hydroxyquinoline N-oxide (NQNO) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB). Biochim Biophys Acta 933: 258-268.

Junesch U and Gräber P (1987) Influence of the redox state and the activation of the chloroplast ATP synthase on proton-transport-coupled ATP synthesis/hydrolysis. Biochim Biophys Acta 893: 275-288.

Junge W, Rumberg B and Schröder H (1970) The Necessity of an electric potential difference and its use for phosphorylation in short flash groups. Eur J Biochem 14: 575-581.

Junge W (1977) Membrane potentials in photosynthesis. Ann Rev Plant Physiol 28: 503-536.

Junge W and Jackson JB (1982) The development of electrochemical potential gradients across photosynthetic membranes. In: Photosynthesis (Govindjee ed) Vol 1 pp. 589-646. Academic Press.

Junge W and Polle A (1986) Theory of proton flow along appressed thylakoid membranes under both non-stationary and stationary conditions. Biochim Biophys Acta 848: 265-273.

Junge W (1987) Complete tracking of transient proton flow through active chloroplast ATP synthase. Proc Natl Acad Sci 84: 7084-7088.

Knaff DB (1989) The regulatory role of thioredoxin in chloroplasts. Trends Biochem Sci 14: 433-434.

Krab K, Hotting EJ, Van Walraven HS, Scholts MJC and Kraayenhof R (1986) Calibration of the carotenoid band shift and the response of the external membrane potential probe oxonol VI with diffusion potentials in (proteo)liposomes and chloroplasts. Bioelectrochem Bioenerg 16: 55-62.

Kramer DM and Crofts AR (1989) Activation of the chloroplast ATPase measured by the electrochromic change in leaves of intact plants. Biochim Biophys Acta 976: 28-41.

Kramer DM and Crofts AR (1990) Demonstration of a high-sensitive portable double-flash kinetic spectrophotometer for measurement of electron transfer reactions in intact plants. Photosynth Res 23: 231-240.

Laasch H, Urbach W and Schreiber U (1983) Binary flash-induced oscillations of [^{14}C]DCMU binding to the photosystem 2 acceptor complex. FEBS-Lett 159: 275-279.

Laszlo JA, Baker GM and Dilley RA (1984a) Chloroplast thylakoid membrane proteins having buried amine buffering groups. Biochim Biophys Acta 764: 160-169.

Laszlo JA, Baker GM and Dilley RA (1984b) Nonequilibration of membrane-associated protons with the internal aqueous space in dark maintained chloroplast thylakoids. J Bioenerg Biomembr 16: 37-51.

Leibl W, Breton J, Deprez J and Trissl HW (1989) Photoelectric study on the kinetics of trapping and charge stabilization in oriented PS2 membranes. Photosynth Res 22: 257-275.

Leibl W and Trissl HW (1990) Relation between the fraction of closed photosynthetic reaction centers and the amplitude of the photovoltage from lightgradient experiments. Biochim Biophys Acta 1015: 304-312.

Lemaire C, Girault G and Galmiche JM (1985) Flash-induced ATP synthesis in pea chloroplasts in relation to proton flux. Biochim Biophys Acta 807: 285-292.

Lill H, Engelbrecht S, Schoenknecht G and Junge W (1986) The proton channel, CF_0, in thylakoid membranes. Only a low proportion of CF_1-lacking CF_0 is active with a high unit conductance (169 fS). Eur J Biochem 160: 627-634.

Malkin R (1987) Photosystem 1. In: The light reactions. (Barber J ed) Elsevier Amsterdam New York Oxford.

McCarthy RE (1979) Roles of a coupling factor for photophosphorylation in chloroplasts. Ann Rev Plant Physiol 30: 79-104.

Melandri BA and Venturoli G (1986) Local and delocalized interactions in energy coupling. In: Encyclopedia of Plant Physiology New Series Vol 19. Photosynthesis III, Photosynthetic membranes and light harvesting systems. (Staehelin LA and Arntzen CJ eds) Springer Verlag, Berlin, Heidelberg.

Morita S, Itoh S and Nishimura M (1982) Correlation between the activity of membrane-bound ATPase and the decay rate of flash-induced 515-nm absorbance changes in chloroplast in intact leaves, assayed by means of rapid isolation of chloroplasts. Biochim Biophys Acta 679: 125-130.

Moss DA and Bendall DS (1984) Cyclic electron transport in chloroplasts. the Q-cycle and the site of action of antimycin. Biochim Biophys Acta 767: 389-395.

Moss DA and Rich PR (1987) The effect of pre-reduction of cytochrome b-563 on the electron-transfer reactions of the cytochrome bf complex in higher plant chloroplasts. Biochim Biophys Acta 894: 189-197.

Nelson-Sigrist K, Sigrist H and Azzi A (1978) Characterization of the dicyclohexylcarbodiimide-binding protein isolated from chloroplast membranes. Eur J Biochem 92: 9-14.

O'Keefe DP (1988) Structure and function of the chloroplast cytochrome b/f complex. Photosynth Res 17: 189-216.

Olsen LF, Telfer A and Barber J (1980) A flash spectroscopic study of the kinetics of the electrochromic shift, proton release and the redox behavior of cytochromes f and b_{563} during cyclic electron flow. FEBS Lett 118: 11-17.

Olsen LF and Barber J (1981) Origin of the slow component of the electrochromic shift: a charge delocalization model. FEBS-Lett 123: 90-94.

Ooms JJJ, Vredenberg WJ and Buurmeijer WF (1989) Evidence for an electrogenic and a non-electrogenic component in the slow phase of the P515 response in chloroplasts. Photosynth Res 20: 119-128.

Ooms JJJ and Vredenberg WJ (1989) Analysis of the slow component of the flash-induced P515 response in chloroplasts. In: Techniques and new developments in photosynthesis research (Barber J and Malkin R eds) Plenum Press New York and London.

Ooms JJJ, Van Vliet PH and Vredenberg WJ (1990a) The slow P515 signal in relation to the status of innermembrane proton domains. In: Current research in photosynthesis. (Baltscheffsky M ed) Vol III pp. 213-216. Kluwer Academic Publishers, Dordrecht, Boston, London.

Ooms JJJ, Versluis W, Van Vliet PH and Vredenberg WJ (1990b) The flash-induced P515 shift in relation to ATPase activity in chloroplasts. submitted for publication.

Ort DR (1986) Energy transduction in oxygenic photosynthesis: An overview of structure and mechanism. In: Encyclopedia of Plant Physiology New Series Vol 19. Photosynthesis III, Photosynthetic membranes and light harvesting systems. (Staehelin LA and Arntzen CJ eds) Springer Verlag, Berlin Heidelberg.

Peters RLA, Bossen M, Van Kooten O and Vredenberg WJ (1983) On the correlation between the activity of ATP-hydrolase and the kinetics of flash-induced P515 electrochromic bandshift in spinach chloroplasts. J Bioenerg Biomemb 15: 335-346.

Peters RLA, Van Kooten O and Vredenberg WJ (1984) The effect of uncouplers (F)CCCP and NH_4Cl on the kinetics of the flash-induced P515 electrochromic bandshift in spinach chloroplasts. FEBS Lett 177: 11-16.

Peters RLA, Van Kooten O and Vredenberg WJ (1985) The kinetics of the flash-induced P515 response in relation to the H^+-permeability of membrane bound ATPase in spinach chloroplasts. J Bioenerg and Biomemb 17: 207-216.

Peters RLA (1986) Electrochromic effects in relation to energy transduction and energy coupling in chloroplast membranes. Thesis, Agricultural University Wageningen.

Petrouleas V and Diner BA (1986) Identification of Q_{400}, a high potential electron acceptor in photosystem 2, with the iron of the quinone-iron complex. Biochim Biophys Acta 849: 264-275.

Petrouleas V and Diner BA (1987) Light-induced oxidation of the acceptor-side Fe(II) of photosystem 2 by exogenous quinones acting through the Q_B binding site. I. Quinones, kinetics and pH-dependence. Biochim Biophys Acta 893: 126-137.

Pfister VR and Homann PH (1986) Intrinsic and artifactual pH buffering in chloroplast thylakoids. Arch Biochem Biophys 246: 525-530.

Polle A and Junge W (1986a) The slow rise of the flash-induced alkalization by photosystem 2 of the suspending medium of thylakoids is reversibly related to thylakoid stacking. Biochim Biophys Acta 848: 257-264.

Polle A and Junge W (1986b) Transient and intramembrane trapping of pumped protons in thylakoids. The domains are delocalized and redox sensitive. FEBS-Lett 198: 263-267.

Press WH, Flannery BP, Teukolsky SA and Vetterling WT (1988) in: Numerical Recipes in C. The art of scientific Computing. Cambridge University Press.

Prochaska LJ and Dilley RA (1978a) Chloroplast membranes conformational changes measured by chemical modification. Arch Biochem Biophys 187: 61-71.

Prochaska LJ and Dilley RA (1978b) Site specific interaction of protons liberated from photosystem 2 oxidation with a hydrophobic membrane component of the chloroplast membrane. Biochem Biophys Res Comm 83: 664-672.

Raines CA and Hipkins MF (1988) Proteolytic enzymes stimulate both the ATP synthesis and hydrolysis functions of the chloroplast ATPase complex. Biochim Biophys Acta 933: 172-178.

Reilly P and Nelson N (1988) Photosystem 1 complex. Photosynth Res 19: 73-84.

Renger G, Kayed A and Oettmeier W (1987) Interaction of halogenated 1,4-benzoquinones with system 2 of photosynthesis. Z Naturforsch 42c: 698-703.

Renger G, Hanssum B, Gleiter H, Koike H and Inoue Y (1988) Interaction of 1,4-benzoquinones with photosystem 2 in thylakoids and photosystem 2 membrane fragments from spinach. Biochim Biophys Acta 936: 433-446.

Rich PR and Moss DA (1987) The reactions of quinones in higher plants. In: The light reactions. (Barber J ed) Elsevier Amsterdam New York Oxford.

Rich PR, Heathcote P and Moss DA (1987) Kinetic studies of electron transfer in a hybrid system constructed from the cytochrome b/f complex and photosystem 1. Biochim Biophys Acta 892: 138-151.

Rich PR (1988) A critical examination of the supposed variable proton stoichiometry of the chloroplast cytochrome bf complex. Biochim Biophys Acta 932: 33-42.

Rich PR (1990) electron and proton transfer mechanisms of the bc_1 and bf complexes: a comparison. In: Current Research in Photosynthesis (Baltscheffsky M ed) Vol III pp. 239-246. Kluwer Academic Publishers, Dordrecht, Boston, London.

Schapendonk AHCM and Vredenberg WJ (1977) Salt-induced absorbance changes of P515 in broken chloroplasts. Biochim Biophys Acta 462: 613-621.

Schapendonk AHCM and Vredenberg WJ (1979) Activation of the reaction 2 component of P515 in chlorplasts by pigment system 1. FEBS-Lett 106: 257-261.

Schapendonk AHCM, Vredenberg WJ and Tonk WJM (1979) Studies on the kinetics of the 515nm absorbance change in chloroplasts. Evidence for the induction of a fast and a slow P515 response upon saturating light flashes. FEBS Lett 100: 325-330.

Schapendonk AHCM (1980) Electrical events associated with primary photosynthetic reactions in chloroplast membranes. Thesis, Agricultural University Wageningen.

Schatz GH and van Gorkom HJ (1985) Absorbance difference spectra upon charge transfer to secondary donors and acceptors in photosystem 2. Biochim Biophys Acta 810: 283-294.

Schreiber U and Rienits KG (1982) Complementarity of ATP-induced and light-induced absorbance changes around 515nm. Biochim Biophys Acta 682: 115-123.

Scheiber U (1986) Detection of rapid induction kinetics with a new type of high-frequency modulated chlorophyll fluorometer. Photosynth Res 9: 261-272.

Schreiber U, Schliwa U and Bilger W (1986) Continuous recording of photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth Res 10: 51-62.

Schreiber U, Klughammer C and Neubauer C (1988) Measuring P700 Absorbance Changes around 830 nm with a new type of Pulse modulation system. Z Naturforsch 43c: 686-698.

Schoenknecht G, Junge W, Lill H and Engelbrecht S (1986) Complete tracking of proton flow in thylakoids - the unit conductance of CF_0 is greater than 10 fS. FEBS-Lett 203: 289-294.

Schuurmans JJ, Peters ALJ, Leeuwerik FJ and Kraayenhof R (1981) On the association of electrical events with the synthesis and hydrolysis of ATP in photosynthetic membranes. In: Vectorial reactions in electron and ion transport in mitochondria and bacteria. (Palmieri F ed) pp. 359-369, Elsevier North-Holland Biomedical Press, Amsterdam.

Selak MA and Whitmarsh J (1982) Kinetics of the electrogenic step and cytochrome b6 and f redox changes in chloroplasts. Evidence for a Q-cycle. FEBS Lett 150: 286-292.

Sigalat C, Haraux F, de Kouchkovsky F, Suong Phung Nhu Hung and de Kouchkovsky Y (1985) Adjustable microchemiosmotic character of the proton gradient generated by systems I and II for photosynthetic phosphorylation in thylakoids. Biochim Biophys Acta 809: 403-413.

Shahak Y, Crowther D and Hind G (1980) Endogenous cyclic electron transport in broken chloroplasts. FEBS Lett 114: 73-78.

Shahak Y (1982) The role of Mg^{2+} in the light activation process of the H^+-ATPase in intact chloroplasts. FEBS-Lett 145: 223-229.

Shahak Y (1985) Differential effects of thiol oxidants on the chloroplast H^+-ATPase in the light and in the dark. J Biol Chem 260: 1459-1464.

Snel JFH (1985) Regulation of photosynthetic electron flow in isolated chloroplasts by bicarbonate, formate and herbicides. Thesis, Agricultural University Wageningen.

Theg SM, Johnson JD and Homann PH (1982) Proton efflux from thylakoids induced in darkness and its effect on photosystem 2. FEBS-Lett 145: 25-29.

Theg SM and Junge W (1983) The effect of low concentrations of uncouplers on the detectability of proton deposition in thylakoids. Evidence for subcompartmentation and preexisting pH differences in the dark. Biochim Biophys Acta 723: 294-307.
Theg SM, Chiang G and Dilley RA (1988) Protons in the thylakoid membrane-sequestered domains can directly pass through the coupling factor during ATP synthesis in flashing light. J Biol Chem 263: 673-681.

Thorne SW, Horvath G, Kahn A and Boardman K (1975) Light-dependent absorption and selective scattering changes at 518 nm in chloroplast thylakoid membranes. Proc Nat Acad Sci USA 72: 3858-3862.
Trebst A (1980) Inhibitors in electron flow: tools for the functional and structural localization of carriers and energy conservation sites. in Methods in Enzymology (San Pietro ed) Vol 69c pp. 675-715. Academic Press New York.
Van Gorkom HJ (1985) Electron transfer in photosystem 2. photosynth Res 6: 97-112.
Van Kooten O, Gloudemans AGM and Vredenberg WJ (1983) On the slow component of P515 and the flash-induced reduction of cytochrome b-563 in chloroplast membranes. Photobiochem Photobiophys 6: 9-14.
Van Kooten O (1988) Photosynthetic free energy transduction Modelling electrochemical events. Thesis Agricultural University Wageningen
Velthuys BR (1981) Electron-dependent competition between plastoquinone and inhibitors for binding to photosystem 2. FEBS Lett 126: 277-281.
Velthuys BR (1987) The photosystem 2 reaction center. In: The light reactions. (Barber J ed) Elsevier Amsterdam New York Oxford.
Vredenberg WJ and Tonk WJM (1975) On the steady-state electrical potential difference across the thylakoid membranes of chloroplasts in illuminated plant cells. Biochim Biophys Acta 387: 580-587.
Vredenberg WJ (1981) P515: A monitor of photosynthetic energization in chloroplast membranes. Physiol Plant 53: 598-602.
Vredenberg WJ, Van Kooten O and Peters RLA (1984) Electrical events and P515 respons in thylakoid membranes. In: Advances in Photosynthesis Research (Sybesma C ed) Vol II pp. 241-246. Martinus Nijhoff/Dr W Junk Publishers, The Hague, Boston, Lancaster.

Vredenberg WJ, Versluis W and Ooms JJJ (1990) Flash-induced absorbance changes in thylakoid membranes in the 490-550 nm wavelength region. The gramicidin-insensitive component. In: Current research in photosynthesis. (Baltscheffsky M ed) Vol II pp. 883-886. Kluwer Academic Publishers, Dordrecht, Boston, London.

Wagner R, Carrillo N, Junge W and Vallejos RH (1982) On the conformation of reconstituted ferredoxin: $NADP^+$ oxidoreductase in the thylakoid membrane. Studies via lifetime and rotational diffusion with eosin isothiocyanate as label. Biochim Biophys Acta 680: 317-330.

White CC, Chain RK and Malkin R (1978) Duroquinol as an electron donor for chloroplast electron transfer reactions. Biochim Biophys Acta 502: 127-137.

Wikström MKF and Saraste M (1984) The mitochondrial respiratory chain. In: Ernster L (ed) Bioenergetics pp. 49-94. Elsevier Amsterdam.

Wise RR and Ort DR (1989) Photophosphorylation after chilling in the light. Effects on membrane energization and coupling factor activity. Plant Physiol 90: 657-664.

Witt HT (1979) Energy conversion in the functional membrane of photosynthesis. Analysis by light pulse and electric pulse methods The central role of the electric field. Biochim Biophys Acta 505: 355-427.

Zimányi L and Garab G (1982) Configuration of the light induced electric field in thylakoid and its possible role in the kinetics of the 515 nm absorbance change. J Theor Biol 95: 811-821.

Zuber H (1987) The structure of light-harvesting pigment-protein complexes. In: The light reactions. (Barber J ed) Elsevier Amsterdam New York Oxford.

Curriculum vitae

Jaap Ooms werd geboren op 31 mei 1958 te Tilburg. In 1977 behaalde hij het diploma Atheneum B aan de Rijksscholen Gemeenschap "Koning Willem II" te Tilburg. Van 1977 tot 1984 studeerde hij biologie (B1g) aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen werd behaald in december 1980. In de doctoraalfase werd een hoofdvakonderzoek uitgevoerd aan de afdeling Chemische Cytologie (Prof. Dr. G. Borst-Pauwels). Bijvakken: Microbiologie (Prof. Dr. G. Vogels) en "toegepaste onderwijskunde voor het hoger onderwijs" (Begeleiding drs. E. Wardenaar en Prof. Giesbers).

Gedurende de periode augustus 1984 tot augustus 1985 was hij als docent biologie verbonden aan het Gertrudislyceum te Roosendaal.

Van september tot december 1985 was hij werkzaam op de afdeling Chemische Cytologie (Prof. Dr. G. Borst-Pauwels) van de Katholieke Universiteit Nijmegen.

Van december 1985 tot december 1989 was hij via een beurs van SON aangesteld aan de vakgroep Plantenfysiologisch Onderzoek van de Landbouwuniversiteit te Wageningen, waar het hier beschreven onderzoek is uitgevoerd onder leiding van Prof. Dr. W.J. Vredenberg.