Electrochromic effects in relation to energy transduction and energy coupling in chloroplast membranes

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Proefschrift
Ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C. C. Oosterlee,
in het openbaar te verdedigen
op dinsdag 16 september 1986
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

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

Stellingen

1. De trage fase in het door licht geïnduceerde P515 signaal die wordt waargenomen na toevoeging van reducerende middelen als duroquinol en dithioniet is niet vergelijkbaar met de trage komponent in het P515 signaal die intrinsiek in geïsoleerde chloroplasten aanwezig is.

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

   Jones, R.W. and Whitmarsh, J. 1985. Photobiochemistry and Photobiophysics, 9. 119-127. Origin of the electrogenic reaction in the chloroplast cytochrome b/f complex.

   Dit proefschrift, hoofdstuk 3.

2. Bij studies naar de initiatie van ATP synthese of hydrolyse door flitsbelichting in donker-geadapteerde chloroplasten dient rekening gehouden te worden met endogene faktoren, zoals de adenylaatkinase aktiviteit, die van invloed zijn op de waarde van de fosfaatpotentiaal die in het donker wordt bereikt.

   Schreiber, U. and Del Valle-Tascon, S. 1982. FEBS Lett. 150. 32-37. ATP synthesis with single-turnover flashes in spinach chloroplasts.

   Dit proefschrift, hoofdstuk 4.

3. De veronderstelling van Morita dat, onder fosforylerende kondities, de afvalsnelheid van de door een lichtflits opgewekte membraanpotentiaal in chloroplasten voornamelijk wordt bepaald door een uitstroom van protonen door de membraan-gebonden ATPase wordt niet door de experimentele resultaten in hoofdstuk 5 ondersteund.

   Morita, S., Itoh, S. and Nishimura, M. 1982. Biochim. Biophys. Acta 679. 125-130. Correlation between the activity of membrane-bound ATPase and the decay rate of flash-induced 515 nm absorbance change.

   Dit proefschrift, hoofdstuk 5.

4. Bij de interpretatie door Selak van de langzame komponent in de opzwaai van het licht-geinduceerde P515 signaal wordt geen rekening gehouden met het feit dat deze opzwaai is geassocieerd met een trage komponent in de afvalkinetiek van het signaal.

Selak, M.A. and Whitmarsh, J. 1982. FEBS Lett. 150. 286-292. Kinetics of the electrogenic step and cytochrome b_6 and f redox changes in chloroplasts.

5. Verschillen in ontwikkelingsstadium van groene planten die bij verschillende lichtintensiteiten worden gekweekt moeten in beschouwing worden genomen wanneer de resultaten van onderzoek naar de moleculaire samenstelling van de chloroplast membranen van deze planten worden geinterpreteerd.

Chapman, D.J., De Felice, J. and Barber, J. 1986. Photosynthesis Research 8. 257-265. Polar lipid composition of chloroplast thylakoids isolated from leaves grown under different light conditions.

Dit proefschrift, hoofdstuk 6.

6. Gezien de verschillen die zijn gevonden met betrekking tot de response tijd van oxonol-VI in verschillende biologische membranen, is het de vraag of deze probe geschikt is voor het interpreteren van de kinetiek van veranderingen in de membraan potentiaal.

Bashford, C.L., Chance, B. and Prince, R.C. 1979. Biochim. Biophys. Acta 545. 46-57. Oxonol dyes as monitors of membrane potential.

7. Het klinisch belang van de vestigingsweerstand (kolonisatieresistentie) tegen pathogene microorganismen voor het infectierisico bij mens en dier, kan pas dan worden vastgesteld wanneer de methoden voor het bepalen van de samenstelling van de inheemse microflora verder zijn geoptimaliseerd.

v.d. Waaij, D. 1979. New criteria for antimicrobial therapy. Eds. v.d. Waaij, D. and Verhoef. J.J. Excerpta Medica ICS 477 Amsterdam-Oxford.

8. Het gelokaliseerde karakter van de schade aan naaldbomen door zure regen in Nederland doet vermoeden dat plaatselijke faktoren hierbij mede een belangrijke rol spelen.

9. Een goede samenwerking tussen bedrijven en universiteiten met betrekking tot het opzetten en verrichten van wetenschappelijk onderzoek wordt steeds belangrijker naarmate de financiele mogelijkheden door de overheid verder worden beperkt.

10. Bij het huidige niveau van de tarieven voor openbaar vervoer zou het begrip "openbaar" ter diskussie kunnen worden gesteld.

11. De kwaliteit en leefbaarheid van woningen gebouwd ten behoeve van de sociale sektor doet vermoeden dat verantwoordelijke architekten en bewindslieden hiermee weinig of geen persoonlijke woonervaring hebben.

12. Gezien de kostenopbouw van receptgeneesmiddelen in Nederland waarbij de apothekersmarge bijna het tienvoudige bedraagt van de winstmarge van de farmaceutische industrie, getuigt het van weinig ekonomisch inzicht van de rijksoverheid om bij het uitvaardigen van kostenbesparende maatregelen de apotheker buiten beschouwing te laten.

Voor mijn ouders,
Delia en Marit

Inhoudsopgave

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Voorwoord

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Abstract

A study was made on the kinetics of the flash-induced P515 electrochromic bandshift signal in spinach leaves and isolated chloroplasts. It was found that part of the signal (i.e. the slow component, also called reaction 2), normally present in dark-adapted membranes is absent from the signal under conditions where the membrane is energized preceding the actinic light flash. This energization can be brought about either by light-driven electron transport or by reverse electron flow caused by ATP hydrolysis. The activation of the chloroplast ATPase was found to be dependent on endogeneous factors determining $\Delta G'ATP$ such as of the adenylate kinase. The acceleration of the overall decay of the flash-induced P515 response found after short periods of illumination was found to be independent of the H^+- permeability of the membrane as has been suggested by others. It is shown that this acceleration can be fully explained by the suppression of the reaction 2 component as a consequence of membrane energization by an activated ATPase. The occurrence of reaction 2 appeared to be dependent on the functional integrity of the membrane and a possible correlation between factors determining the structural organization of the thylakoid and the kinetics of the P515 response was found. It is concluded that the reaction 2 component of the P515 response is the reflection of an intramembranal electrical event, presumably associated with the liberation and subsequent stabilization of protons in inner-membrane domains. It appears that this stabilizing ability is lost upon the addition of a lipophilic protonophore.

Abbreviations

ADP	Adenosine-5′ – diphosphate
ATP	Adenosine-5′ – triphosphate
BSA	Bovine Serum Albumin
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CF0	chloroplast ATPase coupling factor 0
CF1	chloroplast ATPase coupling factor 1
Chl	chlorophyll
cyt b563	b-type cytochrome absorbing maximally at 563 nm
cyt c554	c-type cytochrome absorbing maximally at 554 nm
DCCD	N, N′ – dicyclohexylcarbodiimide
DCMU	3-(3,4-di-chlorophenyl)-1,1-dimethylurea
DGDG	digalactosyldiacylglycerol
DTE	1,4-dithioerythritol
EDTA	ethylenediaminetetraacetic acid
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
Fd	ferredoxin
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LHCP	light-harvesting complex
MGDG	monogalactosyldiacylglycerol
NADPH	reduced nicotinamide-adenine dinucleotide phosphate
OxonolVI	bis(3-propyl-5-oxoisoxazol-4-yl) pentamethineoxonol
P515	pigment complex with absorbance change at 518 nm
P700	trapping center of PS1
P680	trapping center of PS2
PC	plastocyanin
PL	total phospholipids
PQ	plastoquinone
PS1	photosystem 1
PS2	photosystem 2
Qbc	plastohydroquinone-plastocyanin oxidoreductase: cytochrome b-c complex containing bound plastoquinone and Rieske (FeS) protein
R1	reaction 1
R2	reaction 2
Tes	N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid
Tricine	N-(2-hydroxy-1, 1-bis(hydroxymethyl)ethyl) glycine

CHAPTER 1, INTRODUCTION.

1.1 Photosynthesis

Photosynthesis is the unique property of green plants, algae and photosynthetic bacteria to convert light energy into chemical free energy that can be used for biosynthesis. Since organisms that lack photosynthetic capabilities consume or parasite on photosynthetic ones, the process of photosynthesis may be regarded as the basic form of the conversion of energy in nature. In this way, sometimes via a chain of intermediates (carnivorous animals), ultimately all living organisms are dependent on the sun as the ultimate source of energy that is used to maintain life processes.

The overall process of photosynthesis, defined as the light-driven CO_2 reduction with concomitant O_2 evolution, has been known for more than two centuries. As early as 1771, Joseph Priestly implicated the involvement of O_2 and CO_2 when he found that green plants could renew air made bad by the breathing of animals. Few years later, in 1779, a Dutch physician Jan Ingenhousz demonstrated that light was necessary for this purification of air. Nowadays we know that the photosynthetic process proceeds via light and dark reactions. In higher plants and algae the light reactions result in the production of O_2 from H_2O and the formation of two energy-rich products, ATP and NADPH. The dark reactions convert these products into energy rich carbohydrates under fixation of atmospheric CO_2.

1.2 Chloroplast structure and mechanism of photosynthetic energy transduction

Photosynthesis in higher plants and algae takes place in a special organelle, the chloroplast. These organelles possess the unique property of converting light energy into chemical energy. The chloroplast interior consists of a folded structure of thylakoid membranes, part of which are closely packed (granum lamellae). These granum lamellae are interconnected by loose membranes called stroma lamellae. It is now well established (1) that these membranes separate an inner thylakoid phase from the stroma phase. The light reactions occur on the thylakoid membranes that contain light-absorbing pigment complexes, reaction centers and various protein complexes functional in electron transport, hydrogen transfer and phosphorylation of ADP. The matrix of this structure consists of glycolipids,

sulpholipids and few phospholipids. The dark reactions merely take place in the thylakoid-surrounding stroma phase.

An important landmark in the search for the mechanism by which photosynthetic energy conversion leads to $NADP^+$ reduction and ATP production has been the demonstration of the existence of two distinghuishable photoreactions. As originally proposed by Duysens and Amesz (2) these photoreactions, nowadays known to be catalyzed by Photosystem 1 (PS1) and Photosystem 2 (PS2), were shown to operate in series for the production of NADPH. This concept, i.e. linear electron transfer from H_2O to $NADP^+$ can be presented by the so-called Z-scheme (Fig. 1.1). In this scheme, solar

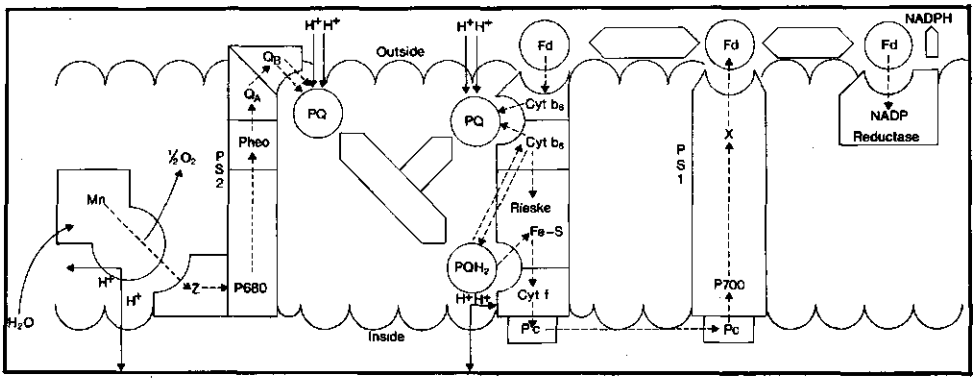

Fig. 1.1. Schematic representation of linear electron transport (- - - →) and coupled proton translocation (——→) across the thylakoid membrane in higher plant chloroplasts. Mn: mangane-containing water splitting protein; P680 and P700: reaction center chlorophylls of photosystem II (PS2) and photosystem I (PS1) respectively; Pheo: pheophytin a; Q_A, Q_B: electron acceptors of PS2; PQ: plastoquinone; cyt f: cytochrome f; Fe-S non-heme iron and sulphur containing Rieske protein; cyt b6: cytochrome b_{563}; Fd: ferredoxin; PC: plastocyanin; X: unidentified electron acceptor for PS1.

energy is primarily captured by the light-harvesting complex (LHCP), a pigment-protein complex primary associated with PS2 that binds up to half of the total chlorophyll a and nearly all chlorophyll b molecules. Light energy absorbed by these antenna pigment molecules is transferred to the pigment-protein complexes containing reaction centre chlorophyll P-700 (PS1) and P-680 (PS2). In the reaction centers of PS1 and PS2, the excited chlorophyll molecules (P700 and P680 respectively) become oxidized with a concomitant reduction of the primary electron acceptors X and QA. These two

light reactions result in membrane charge separation (1.2) and consequently in electron transfer via various electron transfer carriers, like plastoquinone, the Qbc complex containing cytochrome b-563 and cytochrome c-554, Rieske (FeS) protein and plastocyanin. All these connecting compounds undergo reversible oxido-reduction reactions. The strong oxidant that exists after charge transfer in PS2 is capable of oxidizing H2O. Electron transfer from X (PS1) to $NADP^+$ occurs via loosely membrane-bound ferredoxin and via ferredoxin-$NADP^+$ oxidoreductase (3). Because linear electron transfer to $NADP^+$ supplies insufficient ATP required for CO_2 fixation (3.4) the additional ATP is supposed to be produced during cyclic electron transfer (5). During cyclic electron transfer ferredoxin is oxidized by the Qbc redox complex. This results in the back transfer of electrons to the electron transfer chain between the two photosystems.

The mechanism by which electron transfer is coupled to ATP synthesis is still a matter of discussion (6-8). Following the suggestion of Mitchell (9), a vectorial orientation of electron and hydrogen carriers in the membrane will lead to the establishment of a proton motive force (pmf). This pmf, which consists of an electrical ($\Delta\psi$) and a chemical proton activity component (ΔpH) is suggested to be the driving force for ATP synthesis and other energy consuming processes and may be defined according to Mitchell as:

$$\Delta\mu H^+ = -Z\,\Delta\,pH + \Delta\psi$$

in which $\Delta\mu H^+$ is called proton motive force (expressed in mV), Z = RT/F (J/C, mV), ΔpH is the pH gradient (pH units) and $\Delta\psi$the electric potential (mV).

Many experimental data have supported the validity of the chemiosmotic theory for energy coupling in chloroplast membranes (10,11). For example, in chloroplasts, the coupling of proton uptake with electron transfer was already noticed in 1964 by Jagendorf (12), who also reported the fact that a pre-established transmembrane proton gradient could drive ATP synthesis (13). Similar effects of pH gradients and light on Pi-ATP exchange reactions (14) and conformational changes in CF1 (15) have been established. Reversed electron flow caused by a pH gradient was observed by Schreiber and Avron (16). However, after finding contradictory stoichiometries between the numbers of electrons, protons and ATP molecules involved (17),

serious doubts, in particular about the simplicity of this hypothesis arose.

An alternative model on the mechanism of coupled electron transport was proposed by Williams (18). In this model the importance of electron transport mediated accumulation of protons in hydrophylic pockets of the membrane was emphasized. In this model, the coupling between redox and ATPase activities would be mediated by charge separations and proton displacements in restricted domains inside or in very close vicinity of the membrane. Such a model could explain ATP formation in the absence of either a transmembrane proton gradient or a transmembrane potential as was observed by Ort et al. (19). At this time, a challenging question is whether the coupling of ATP synthesis to electron flow occurs by means of a true bulk to bulk transmembrane ΔpH or by a mere localized transmembrane or short range proton gradient (8). Models have been presented in which protons are displaced via the membrane-water interface (8) or even through pathways within the membrane (20). However, since no convincing evidence for the proposed mechanisms is available until now, the chemiosmotic hypothesis still is the most coherent framework to understand and predict many facets of the coupling mechanism.

1.3 The charge separation upon the excitation of the photosynthetic reaction centers in relation to energy conversion

A detailed review on this matter has been given by Witt (21). The magnitude of the electrical potential in a single-turnover light flash has been measured directly by microelectrodes (22) and indirectly, making use of the absorbance response of an intrinsic probe, the P515 pigment complex, as well as extrinsic probes, such as oxonol VI. Using these methods variable results have been reported with regard to the magnitude as well as to the kinetic profile of the flash-induced electrical potential. However, general agreement exists that these probes are useful instruments for studying the magnitude and kinetics of formation and decay of transmembrane electric potential of the inner chloroplast. The light-induced absorbance change at 518 nm (ΔA518), in photosynthetic membranes of plants and algal cells, originally detected by Duysens (23) has been evidence to be linearly related to a homogeneous transmembrane electric field (21,24,25). There is now agreement that ΔA518, can be ascribed to an electrochromic absorbance

shift of a pigment moiety called P515. The P515 absorbance signal in single turnover flashes is a multi-phasic phenomenon, reflected by a biphasic rise and a decay which consists of at least three different single exponentials (26). The biphasic rise first was observed by Joliot and Delosme (27) in chlorella.

In addition to a prompt (0.1 μs) rise (21), a slower (5-50 ms) rise has been observed. These two phases have been called phase a and phase b respectively. Whereas the fast rise is obviously associated with charge separation in the photosynthetic reaction centers, the origin of the slower component is still a matter of debate. Olson and Barber (28) suggested that it results from delocalization of the fast electric field. It also was suggested to result from redox reactions in the Qbc region of the electron transfer chain (29-32). An association with cyt.b6 reoxidation has been suggested by Selak and Whitmarsh (33). However, recently it was shown (34) that the seeming correspondence between the potential associated with this secondary electron transport and a turnover of cyt-b6 only holds for a single flash. A second turnover of cyt-b6 induced by a second flash, given 100 msec after a first one does not, ir at least much less, cause a slow rise in the flash-induced P515 response. Moreover, the occurrence of a slow phase in the rise of the P515 response is obligatory associated with a slow phase in the decay kinetics. The interpretation of Selak does not account for the fact that the field generated by this secondary electron transfer decays with a half time which is considerably higher than the decay of the potential generated by electron transfer through the reaction centers (26). The occurrence of biphasic decay kinetics as described by Rumberg and Siggel (35), Junge et al. (36) and Girault and Galmiche (37), originally was found under phosphorylating conditions. It was interpreted to be due to an increased proton conductance through the ATP synthesizing enzyme complex above a critical potential. On the other hand, it was reported by Schapendonk (38) that the decay rate is dependent on the membrane integrity rather than on the membrane conductance.

An alternative interpretation was suggested by Schapendonk et al. (26). According to analysis, the complex and multi-phasic rise and decay kinetics of the flash-induced P515 response is interpreted to be the composite result of at least two different responses called reaction 1 and reaction 2. Reaction 1, characterized by a fast (ns) rise and a single exponential decay ($k=10\ s^{-1}$) is suggested to reflect the generation and

decay of the delocalized transmembrane electric field induced by the light induced charge separation in PS1 and PS2. This reaction is observed without interference from the other reaction components in aged chloroplasts or in chloroplasts which have been rethawed from a frozen preparation. In all cases where reaction 1 is the exclusive component, the first order rate constant of the decay titrates linearly with the concentration of permeant ions in the absence and presence of ionophores. In contrast to reaction 1, reaction 2 is never observed in the absence of the other reactions. Consequently, the response of reaction 2 can only be obtained by subtracting the other components from the overall P515 response. Methods of measuring these components have been described by Schapendonk (26). Reaction 2 is characterized by a relatively slow rise, occurring in 20-100 ms and a decay with a first order rate constant of the order of 1 s^{-1}, i.e. a morefold of the rate constant of the decay of reaction 1. Its occurrence is strictly dependent on the integrity of the membrane, i.e. it disappears upon ageing and is absent in chloroplasts that have been rethawed from a frozen preparation. It has been proposed that reaction 2 is caused by a lateral and transversal delocalization of inner membrane electric fields associated with the liberation and subsequent stabilization of protons in inner membrane domains near the Fe-S cyt-bf protein complex (39). This transversal delocalization of inner-membrane localized fields occurs under, as yet unknown, conditions which promote the formation of hydrophillic innermembrane microcompartments. Protons may be stabilized in these compartments and are hindered in their transversal and lateral diffusion. The consequence of hindered diffusion as suggested by others (40) on the formation of isopotentials under continuous illumination have been simulated in a model (41). There appears a reasonable agreement with the experimental data and the simulation model. These domains might be connected via lateral H-conductive channels with other membrane domains i.e. the ATP synthetase. In this respect it is of interest to mention that, in conformation with the results of others (42,43), reaction 2 can also be induced in the dark by ATP driven reversed electron flow. It has been convincingly demonstrated that the slow light-induced and ATP-hydrolysis induced electric potential are of the same nature and in fact fully complementary (42,43,44). Thus, with respect to reaction 2 there is accumulating evidence that it has its origin in innermembrane phenomena which are dependent on the existence of domains which facilitate proton interaction in the lateral plane of the

membrane and may give rise to localized chemiosmotic coupling mechanisms, and electrochromic effects on the native P515 pigment complex.

1.4 Outline of this thesis

This thesis deals with studies that are performed to obtain more information about the nature and physiological significance of the slow component (i.e. reaction 2) of the flash-induced P515 response. The results will be discussed in relation to the dynamic function of the thylakoid membrane in energy transduction and energy coupling.

In chapter 3 the results of a comparative study of the effect of preillumination on the kinetics of the P515 response and on the activity of the chloroplast ATPase are presented. It gives the results of the effect of energization of the membrane by different methods on the kinetics of the P515 response in intact leaves, isolated chloroplasts and isolated well-coupled photosystem-1-enriched subchloroplast vesicles.

Chapter 4 describes the effect of illumination of isolated chloroplasts with single-turnover flashes on the chloroplast ATPase. Different effects of these short periods of illumination on the activity of the chloroplast ATPase are discussed in relation to the activity of the enzyme adenylate kinase, in two different types of chloroplasts.

In chapter 5 experiments are presented on the kinetics of the flash-induced P515 response in relation to the H^+-permeability of the membrane-bound ATPase in chloroplasts.

In chapter 6 the results are presented of a study of the kinetics of the P515 response in relation to the lipid composition of the thylakoid membrane. These results may indicate a possible correlation between structural organization of the thylakoid membrane and the kinetics of the flash-induced P515 response.

Chapter 7 deals with the effects of uncouplers of photophosphorylation on the kinetics of the flash-induced P515 response and on the electron transport rate. The differences in effect between uncouplers that act as proton carriers in the membrane (i.e. CCCP and FCCP) and uncouplers that act as a proton binding agent in the hydrophilic phase (i.e. NH_4Cl) on the kinetics of the P515 response are discussed.

Most of the results presented in this thesis have been published (or

submitted) as indicated in the footnotes of the chapters.

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CHAPTER 2, MATERIALS AND METHODS.

In each of the following chapters details about material (preparations) and specific methods are given. In this chapter I restrict myself to some general aspects.

2.1 Cultivation of plants

Two cultivars of Spinacia oleracea have been used. One summer type spinach cv: Breedblad and one winter type spinach cv: Bergola. A soil mixture of commercial soil (TKS : Torf-Kultur-Substrat) and gardener soil with the following composition; 5 parts compost + 5 parts leafmould, 3 parts peat dust + 2 parts stable manure, and 1 part sand was used in a proportion of 1 : 1. Spinach was sowed in fresh soil in trays of 60 x 80 x 20 cm. After the seeds were put in furrows, a little fungicide was added. Plants were exposed to a light period of 8 hours per day. During the light period the plants were placed in a greenhouse where they received supplementary light from high-pressure mercury lamps (Philips HPLR 400). Minimal light intensity on the spinach leaves was kept at 80 $W.m.^{-2}$. Heat radiation from the lamps was dissipated by a waterfilter of 7 cm thickness through which water was flowing at a rate of minimal 5 liters an hour. The temperature at the soil surface and on the leaves was 16^{o} C and 18^{o} C respectively. The temperature never exceeded 20^{o} C. The relative humidity of the atmosphere was kept at 70%. Airflow above the leaves was prevented, in order to reduce changes in texture of the leaves which makes it harder to isolate intact chloroplasts. Insecticides were found to have an influence on the quality of the isolated chloroplasts. Since most insecticides affect the dynamic function of biological membranes, effects on the kinetics of the P515 response can be expected. If plants were to be treated with insecticides, a period of at least 4 days was taken before harvesting. Plants of about 5 weeks old were used for chloroplast isolation.

2.2 Isolation of chloroplasts

Leaves (about 30 grams) were harvested in the morning after 2 hours light. Leaves were brought into a cold room (temperature about 10^{o} C) with low green light irradiation. The midribs were cut out of the leaves which subsequently were cut into square pieces of about 2 x 2 mm. These pieces were deposited in 50 ml of grinding medium containing: 0.33 M sorbitol, 10

mM Na2P2O7, 5 mM MgCl2 and 2 mM ascorbate. pH was 6.5 (HCL). The leaf pieces were ground by an Ultra-Turrax (type: TP 18/10, 20000 rpm, Janke and Kunkel KG). The turrax gave grinding pulses of ½ sec. The leaf suspension received 4 of these pulses. In between the pulses the suspension was manually shaken. The ground suspension was filtered through 4 layers of perlon cloth (mesh distance 40 m). The cloth was immersed in cold grinding medium before the filtering. The filtered suspension was put into two 50 ml perspex centrifuge tubes. The tubes had a flat bottom and were highly polished on the inside. In a chillspin table top centrifuge the debris was spinned down in 50 sec at 2800 rpm. The pellet was resuspended by gently shaking with 1 ml of washing medium containing: 0.33 M sorbitol, 50 mM Hepes, 1 mM MgCl2, 2 mM EDTA, 2 mM ascorbate and 0.1 mg/ml BSA. pH 7.5 (NaOH). When the bottom of the tubes was clear, the rest of the 50 ml washing medium was added. Chloroplasts were spinned down again for 60 sec at 2800 rpm. The pellet was resuspended with 0.5 ml of stock medium containing: 0.33 M sorbitol, 50 mM Hepes, 1 mM MnCl2, 3 mM MgCl2, 2 mM EDTA, pH 7.5 (NaOH). Resuspension was done by shaking gently. The stock solution was kept in the dark on ice. This procedure routinely yielded preparations with 90 to 95% intact chloroplasts as determined by ferricyanide reduction (1). Broken chloroplasts (uncoupled) gave electron transport rates of about 250 mol O2/mg chl.hr. The coupling ratio of chloroplasts was between 5 and 10.

2.3 Flash-induced absorbance difference measurements of P515

Flash-induced absorbance changes were measured in a modified Aminco-Chance absorption difference spectrophotometer as described by Schapendonk (2). The apparatus operated in the dc single-beam mode. A 1.0 x 1.0 cm sample cell was inserted into a special holder that could be thermostated. Leaf and chloroplast measurements were performed at 3° C. Samples were dark-adapted at 3° C for at least 1 hr before measuring. Saturating single-turnover actinic flashes with a half-width of 8 sec and a wavelength above 665 nm were transmitted to the sample via light guides. The output voltage of the photomultiplier, linear with the intensity of the 518 nm measuring beam transmitted by the sample, was fed into a differential amplifier (Tektronix 5A22). After compensation the amplified output signal was sampled and averaged on a Minc-11 minicomputer (Digital Equipment Co.) with a limiting time constant of 0.3 msec. The photomultiplier was shielded from

actinic light by an appropriate filter combination (Wratten 58, BG 38 and BG 18). Single flashes or a group of 2 to 6 flashes with time intervals of 100 msec were fired. The repetition rate usually was 0.05 Hz. A number of 6 to 12 absorbance responses were sampled and averaged. Analysis of the kinetics of the flash-induced P515 response was performed as described by Schapendonk (2). Curve analysis into the exponential phases was done according to an adopted method (3) with the help of a computer assisted calculation programme. Deconvolution of the overall response into reaction 1 and reaction 2 is described in chapter 3.

2.4 Measurement of ATP-hydrolysis and synthesis

ATP-hydrolysis and synthesis in chloroplasts was determined with the bioluminescent firefly luciferin-luciferase assay method in an appropriate monitoring device (4), equiped with a photomultiplier. Details of the methods are described in chapter 3.

2.5 Determination of adenylate kinase activity

Changes in ATP concentration due to adenylate kinase activity were measured with the luciferine-luciferase luminescence assay. Details of the methods are presented in chapter 4.

2.6 Determination of membrane lipid composition

For the determination of the lipid composition of the thylakoid membrane, envelope-free thylakoids were prepared according to a modified method of Douce (5) as described in chapter 6. Extraction, separation, staining and identification of the different membrane lipids was done according to documented description. Details are given in chapter 6.

2.7 Spectral measurements

Spectral measurements for the determination of total chlorophyll content, the molar concentration ratio of chlorophyll and P700, chlorophyll and cytb563 and chlorophyll and cytf were done with an Aminco DW-2A spectrophotometer. Details are given in chapter 6.

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CHAPTER 3, ON THE CORRELATION BETWEEN THE ACTIVITY OF ATP-HYDROLASE AND THE KINETICS OF THE FLASH-INDUCED P515 ELECTROCHROMIC BANDSHIFT IN SPINACH CHLOROPLASTS.

In collaboration with M. Bossen, O. van Kooten and W.J. Vredenberg. Modified version of publication in Journal of Bioenergetics and Biomembranes, Vol. 15, No. 6, (1983) 335-346.

Study on photosystem-1-enriched subchloroplast vesicles in collaboration with F.A.L.J. Peters, R.H.M. van der Pal, W.J. Vredenberg and R.J. Kraayenhof. Modified version of part of publication in Biochimica et Biophysica Acta, 766 (1984) 169-178.

Abstract

The P515 absorbance change upon single-turnover light flashes has been studied in intact leaves, isolated chloroplasts and isolated photosystem-1-enriched subchloroplast vesicles from spinach. A comparative study of the effects of preillumination on the kinetics of the P515 response and on the activity of the chloroplast ATPase has been made. The slow component (reaction 2) in the flash-induced P515 response normally present in dark-adapted chloroplasts is reduced or even absent under conditions in which the ATPase is activated by preillumination. Qualitatively the same results were found in subchloroplast vesicles following energization of the membrane by either preillumination or by a transmembrane Δ pH. This suppression of reaction 2 appeared to be temporary in leaves, chloroplasts and subchloroplast vesicles; its duration in chloroplasts is shown to be dependent on the amount of ATP present. Tentoxin inhibits the preillumination dependent suppression of reaction 2 in chloroplasts.

3.1 Introduction

The P515 electrochromic bandshift (for a review see ref. 1) following a single saturating light flash in dark-adapted and well-preserved chloroplasts shows multiphasic rise and decay kinetics (2,3). These kinetics cannot be explained in terms of generation and decay of a transmembrane electric field. Such a field would be expected to follow a single exponential dark decay with a rate constant determined by the membrane capacitance and the membrane conductance, provided these are field-independent (4). The decay rate, however seems to be dependent on the membrane integrity rather than on the membrane conductance (3). According to Schapendonk (3) the decay of the P515 absorbance change in intact chloroplasts is almost exclusively determined by the decay of a special type of electrochromic change (called reaction 2) that is not directly linked to a transmembrane potential but rather to an intramembranal local field in the vicinity of the P515 complex. It has been suggested (5,6) that this local, intramembranal field may involve changes in surface charges on the CF1 part of the ATPase complex. In spite of the fact that the origin of the slow component (reaction 2) is still unclear, it must be stated that the occurrence of multiphasic rise and decay kinetics of the P515 bandshift obviously depends on

the state of activation of the chloroplast ATPase (7-10).Recently, a P515 electrochromic shift has been shown to occur in the dark following the addition of ATP to a suspension of light-activated chloroplasts (11,12). Moreover, there seems to be a strict complementarity of the ATP-induced and the light-induced P515 shift in chloroplasts with respect to the slow phase (11).

In this communication we present results that support the idea that the activity of the chloroplast ATPase is reflected in an alteration in the kinetics of the P515 bandshift. Activation of the chloroplast ATPase by short periods of illumination in intact leaves or intact chloroplasts results in a complete suppression of reaction 2 from the P515 shift induced by a subsequent light flash. The same results are obtained with broken chloroplasts after short periods of illumination in the presence of DTE and ATP. In both cases, a single saturating light flash following a period of preillumination results in an electrochromic shift characterised by a fast rise and a subsequent single exponential dark decay with a half-time of approximately 75 msec. The suppression of reaction 2 caused by preillumination appears to be reversible. The duration of the suppression is dependent on the amount of ATP present in the sample. Broken chloroplasts in the absence of DTE and ATP appear to be completely insensitive to preillumination. Inhibition of the ATPase by the addition of 2.5 µmol/liter tentoxin completely prevents the suppression of reaction 2 by preillumination in intact chloroplasts as well as in broken chloroplasts in the presence of ATP and DTE.

Qualitatively the same results were found for photosystem-1-enriched subchloroplast vesicles. Also in these preparations it was observed that the recovery of the slow P515 response after a period of 20 sec illumination or after preacidification of the vesicle lumen followed by an upward pH jump takes about 100 sec whereas the fast P515 response is not altered significantly.

3.2 Material and Methods

Freshly grown spinach (Spinacia Oleracea cv: Bergola) was used for all experiments. The plants were grown under high-pressure mercury lamps (Philips HPLR 400) at an intensity of approximately 80 W/m^2 with a light period of 8 hours per day. Provisions were made to keep the temperature at the

leaf and soil surface at 18-20° C. The relative humidity of the atmosphere was minimal (70%). Intact chloroplasts were routinely isolated according to a modified method of Walker (13) as described in chapter 2. This procedure routinely yielded preparations with 90-95% intact chloroplasts as determined by ferricyanide reduction (14). Broken chloroplasts were obtained by a 60-sec osmotic shock on ice, in a medium containing 5 mmol/liter $MgCl_2$ and 10 mmol/liter Tricine adjusted to pH 7.8 with NaOH, and subsequent addition of double-strength assay medium which contained 660 mmol/liter sorbitol, 2 mmol/liter $MgCl_2$, 2 mmol/liter $MnCl_2$, 4 mmol/liter EDTA, and 100 mmol/liter Hepes, adjusted to pH 7.8 with NaOH. The Hill reaction rate in freshly broken and uncoupled chloroplasts was determined to be minimal 200 µmol O_2/mg chl./h. The ratio of electron-transport activities of NH_4Cl-uncoupled and coupled chlorolasts was determined to be minimal 5. Chlorophyll content was determined spectrophotometrically (15). Absorbance changes at 518 nm induced by single-turnover flashes in either intact leaves or isolated chloroplasts were measured in a modified Aminco chance absorption difference spectrophotometer as described by Schapendonk (16) (see chapter 2). Leaf and chloroplast measurements were performed at 3° C. Samples were dark-adapted at 3° C for at least 1 h before measuring. Saturating single-turnover actinic flashes with a half-width of 8 µsec were transmitted to the sample via light guides. Preillumination with red light came from a 250-W tungsten lamp and was transmitted to the sample via light guides. ATP hydrolysis in chloroplasts was determined with the bioluminescent firefly luciferin-luciferase assay method. Chloroplasts were illuminated with light of wavelengths above 665 nm. Immediately after cessation of the illumination, 200 µl luciferin-luciferase (LKB Wallac) was added to the sample. The light emission associated with the ATP-hydrolyzing luciferin-luciferase reaction was determined by a photomultiplier. Calibration was carried out by titrating known amounts of ATP. This assay method was linear up to 10^{-6} mol/liter ATP with a lower detection limit of 10^{-11} mol/liter ATP. PS-1-enriched vesicles were isolated from market spinach as described by Peters (17). A pH jump was performed by adding 20 µl 0.2 M succinic acid to the reaction mixture (final pH 5.5), followed after 20 sec of equilibration by addition of 20 µl 0.4 M NaOH (final pH 7.8).

3.3 Results and Interpretation

A representative example of the time course of the absorbance change at 518 nm (ΔA518) upon a single-turnover light flash in dark-adapted intact chloroplasts is illustrated in Fig. 3.1 (dashed curve). From this figure it

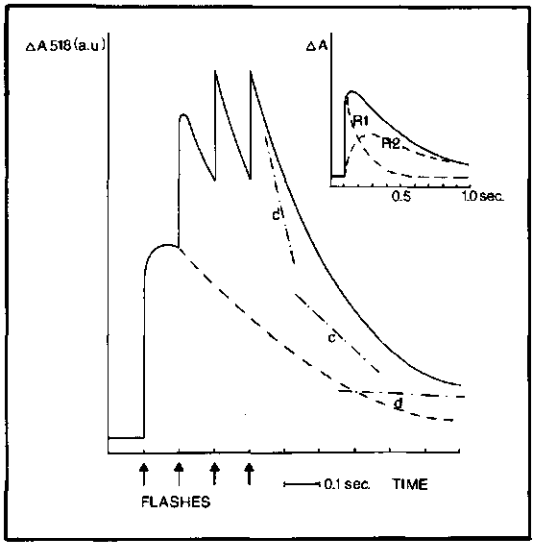

Fig. 3.1. Absorbance changes at 518 nm in dark-adapted intact chloroplasts induced by a single flash (dashed curve) and by a series of four flashes fired at 100-msec time intervals (solid curve). Average of 10 series of flashes fired at a rate of 0.10 Hz. The decay kinetics of the third and subsequent flashes are identical and consist of three single exponentials, representative for phases c', c, and d (dot-dash curve). The insert shows the deconvolution of the overall signal induced by a single flash into reaction 1 and reaction 2. The maximal ΔA518 (ΔI/I) reached in a single saturating light flash was approximately 8×10^{-3}.

can be seen that ΔA518 occurs with complex multiphasic rise and decay kinetics. Analysis of the semilogarithmic plots of these changes (3) has revealed five different phases in the kinetics. An initial fast absorbance increase, called phase a, completed within a time shorter than the resolution time of the measuring system (0.3 msec), is followed by a relatively slow increase in absorbance in the first 20-70 msec. According to this analysis, the subsequent decrease in absorbance appears to be composed of three different phases. A major slow phase c with a half-life of about 400 msec is preceded by a fast phase c' with a half-life of about 75 msec and followed by a small phase d decaying with a half-life of about 1500 msec. These data have been interpreted as an indication that the phase c' decay is associated with the initial fast rise (16). This interpretation is corroborated by data of the absorbance response in a series of four consecutive flashes, fired at time intervals of 100 msec as shown in Fig. 3.1. The decay kinetics of the response after the third and subsequent flashes were found to be identical, and could be characterized by three single

exponential decay phases equal to phases c', c, and d. According to Schapendonk and Vredenberg (3) phases a and c' have been ascribed to a reaction called reaction 1. Thus, with the determination of phase a (1st flash) and phase c' (4th flash), the response of reaction 1 could be determined (inset, Fig. 3.1). The response obtained by subtracting reaction 1 from the overall P515 response upon a single flash has been attributed to that of reaction 2 (inset, Fig. 3.1). The rise kinetics of reaction 2 show a slow absorbance increase within 150 msec. The decay of reaction 2 after the flash is biphasic, with rate constants determined by the characteristic relaxation times of phases c and d. The addition of 5 µmol/liter gramicidin to a sample of intact chloroplasts was found to abolish reaction 2 and, as expected, to enhance the decay rate of reaction 1 (phase c'), as can be seen from Fig. 3.2. Surprisingly, the addition of gramicidin did not affect

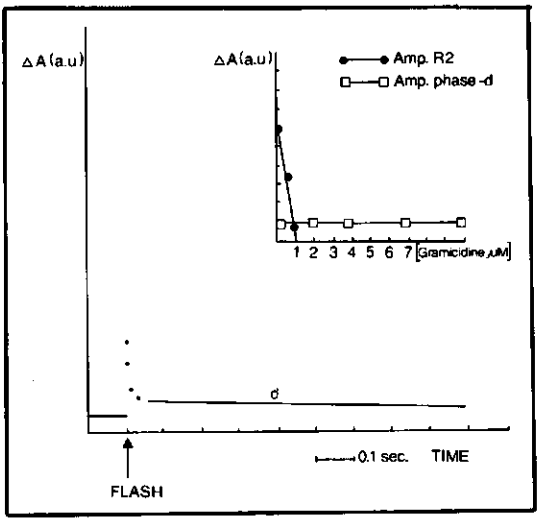

Fig. 3.2. Absorbance changes at 518 nm in dark-adapted intact chloroplasts induced by a single flash in the presence of 3 µmol/liter gramicidin. The magnitude of the initial rise is virtual because of the rapidity of the decay and the 5-msec time resolution at which it was measured. Average of 10 single flashes fired at a rate of 0.10 Hz. The insert shows the effect of gramicidin on the amplitudes of reaction 2 and phase d.

phase d (inset, Fig. 3.2). This result, in combination with the observation (3) regarding the dissimilarity of the spectrum of phase d compared to the spectrum of P515, has strengthened the conclusion that phase d is not due to an electrochromic response of the P515 pigment complex. Phase d may be due to a change in light scattering. The contribution of phase d to the overall signal of P515 is about 8% in intact chloroplasts and intact leaves, and is negligibly small in broken chloroplasts. Therefore, reaction 2 in intact chloroplasts and intact leaves needs a small correction for the virtual absorbance change at 518 nm associated with phase d. After this

correction is made, the kinetics of the P515 response in intact leaves, intact chloroplasts, and broken chloroplasts are found to be similar.

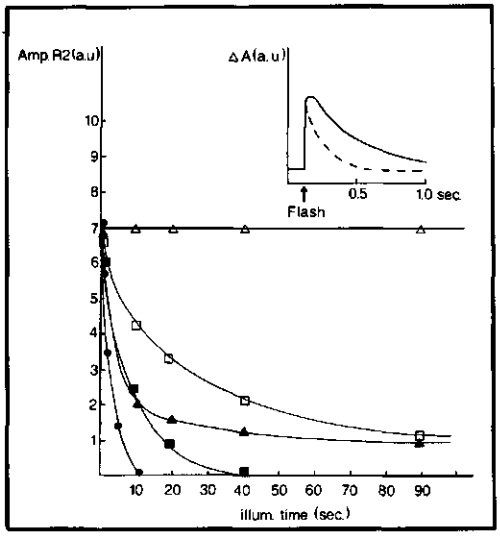

Fig. 3.3. The effect of preillumination on the amplitude of reaction 2 of the flash-induced P515 response in intact leaves (●), intact chloroplasts (■), and broken chloroplasts in the absence (Δ) and presence (□) of 300 μmol/liter ATP. The effect of preillumination in broken chloroplasts in the presence of 300 μmol/liter ATP and 1 mmol/liter DTE is also shown (▲). The inset shows the P515 response induced by a single light flash in intact chloroplasts before (solid curve) and after (dashed curve) a preillumination period of 40 sec. 1 a.u. corresponds to approximately 8×10^{-4} ($\Delta I/I$).

Figure 3.3 shows the effect of preillumination on the amplitude of the reaction 2 component in the P515 response, induced by a light flash given 5 sec after an illumination period in intact leaves, intact chloroplasts, and broken chloroplasts. It shows that the illumination of intact leaves for 10 sec results in the complete suppression of reaction 2 from the flash-induced P515 response. The light flash causes only a reaction 1 type electrochromic shift characterized by a fast rise and a single exponential dark decay (inset, Fig. 3.3). The remaining reaction 1 type electrochromic shift is insensitive to more prolonged periods of illumination (data not shown). An identical effect of preillumination is found for a suspension of intact chloroplasts, although the duration of the light period required for a complete suppression of reaction 2 appears to be longer (40 sec). The suppression of reaction 2 from the overall signal appears to be temporary and can be completely overcome by a period of dark adaption preceding the light flash. From Fig. 3.4 it can be seen that after a dark period of 20 min following a period of 60 sec illumination, complete recovery of reaction 2 in a subsequent light flash occurred in leaves and intact chloroplasts. Surprisingly, broken chloroplasts in the absence of DTE and ATP are insensitive to preillumination (see Fig. 3.3). In these samples, the P515 response induced by a single saturating light flash cannot be altered

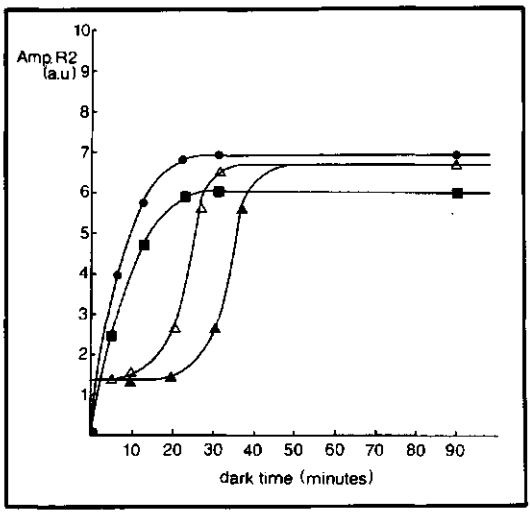

Fig. 3.4. The effect of dark adaptation after a 60-sec preillumination on the recovery of the amplitude of reaction 2 in intact leaves (●), intact chloroplasts (■), and broken chloroplasts in the presence of 1 mmol/liter DTE, with 100 µmol/liter ATP (▲) or with 300 µmol/liter ATP (▲), respectively. 1 a.u. corresponds to approximately 8 x 10^{-4} ($\Delta I/I$).

significantly by a period of preillumination, neither with respect to reaction 1 nor with respect to reaction 2. The addition of ATP and DTE to these samples preceding the period of illumination, however, resulted in a significant (70%) suppression of reaction 2 in a subsequent light flash. In accordance with the results mentioned earlier for intact leaves and intact chloroplasts, this suppression of reaction 2 from the overall signal is reversible. The duration of the period of dark adaption following the period of illumination and required for a full recovery of reaction 2 in a subsequent light flash appears to be dependent on the amount of ATP present in the sample (Fig. 3.4). As can be seen from Table I, illumination of intact chloroplasts, as well as broken chloroplasts in the presence of ATP and DTE, results in an activation of the latent ATPase, whereas the ATPase in broken chloroplasts in the absence of DTE and ATP cannot be activated. These results suggest evidence for a close connection between the kinetics of the P515 shift and the state of activation of the chloroplast ATPase. Under conditions in which the chloroplast ATPase is activated and ATP hydrolysis can be measured, the slow component of the P515 shift induced by a single light flash is largely if not completely suppressed. This suppression is demonstrated to be reversible, and the duration of the suppression period appears to be dependent on the amount of ATP present in the sample. Figure 3.5 shows the effect of preillumination on the kinetics of the P515 response in intact chloroplasts in the presence and absence of 2.5 µmol/

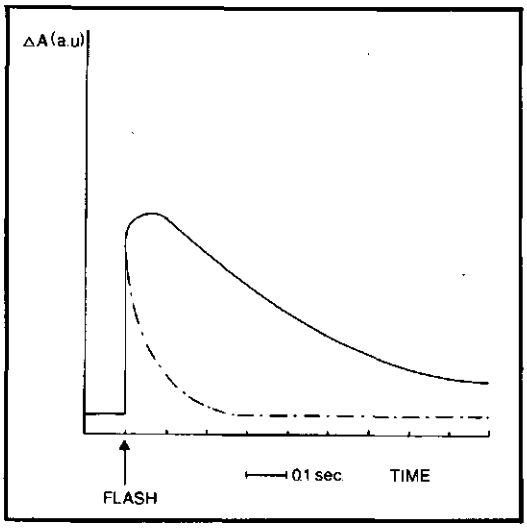

Fig. 3.5. The effect of a preillumination period of 120 sec on the kinetics of the flash-induced P515 response in intact chloroplasts in the presence (solid curve) and absence (dot-dash curve) of 2.5 μmol/liter tentoxin. The maximal ΔA518 (ΔI/I) reached in a single saturating light flash was approximately 8×10^{-3}.

liter tentoxin. In the presence of tentoxin, preillumination of the sample has no effect on the kinetics of the P515 response induced by a subsequent light flash. Tentoxin is known to be a strong inhibitor of the CF1 part of the ATPase (18). Accordingly no ATP hydrolysis was measured in the samples in the presence of tentoxin (see Table I). Qualitatively the same results were found for photosystem-1-enriched subchloroplast vesicles. In Fig. 3.6,

Table I. The Rate of ATP Hydrolysis (mol ATP/mg chl./min.) in Dark-Adapted and Light-Activated Intact and Broken Chloroplasts.*

	Dark-adapted:	After 60 sec. illumination in the presence of:					
	No addition	No addition	100 μmol/l ATP	1 mmol/l DTE	1 mmol/l DTE + 2.5 μmol/l tentoxin	100 μmol/l ATP + 1 mmol/l DTE	100 μmol/l ATP + 1 mmol/l DTE + 2.5 μmol/l tentoxin
Intact	n.h.d.*	7×10^{-8}		6×10^{-8}	n.h.d.		
Broken	n.h.d.	n.h.d.	4×10^{-8}			4×10^{-8}	n.h.d.

* = no hydrolysis detectable i.e. less than 10^{-11} mol ATP/mg chl./min.

*Light-activation was performed under conditions mentioned in the table. Hydrolysis was measured in the presence of 100 μmol/liter ATP which, if not present during preillumination, was added before measurement. The rate of hydrolysis in intact chloroplasts refers to the rate measured after breaking the chloroplasts immediately after the pretreatment.

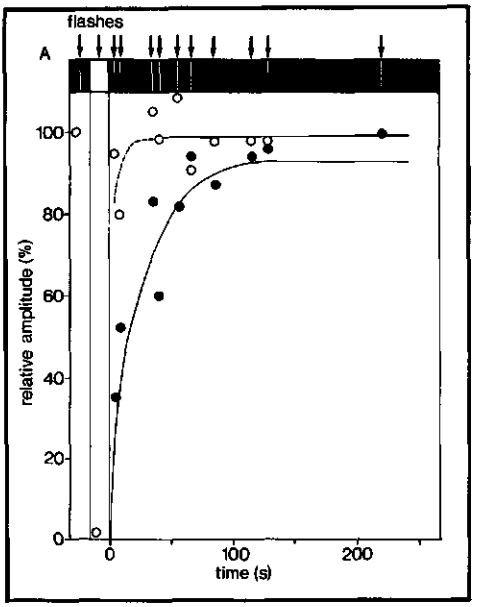

Fig. 3.6. Effect of preillumination on the relative amplitudes of reaction 1 (o——o) and reaction 2 (●——●) of the flash-induced P515 absorbance change in isolated photosystem-1-enriched sub-chloroplast vesicles. Flashes were fired before, during and after a 20 sec period of continuous illumination as indicated. Reaction medium contained; 5 mM Tes-KOH buffer (pH 7.6), 2.5 mM KH_2PO_4, 25 mM NaCl, 25 mM KCl, 5 mM $MgCl_2$, ferredoxin 4 μM and NADPH 0.5 mM. Chlorophyll concentration 50 μg/ml.

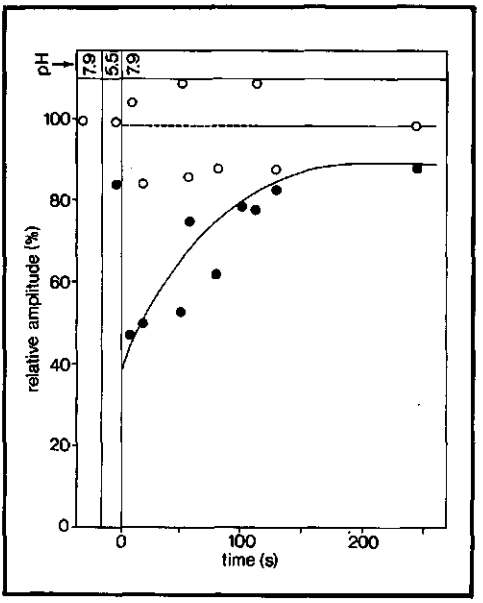

Fig. 3.7. Effect of an acid-base transition on the relative amplitudes of reaction 1 (o——o) and reaction 2 (●——●) of the flash-induced P515 absorbance change in isolated photosystem-1-enriched subchloroplast vesicles. Conditions as in Fig. 3.6.

the normalized amplitudes are given of the fast (reaction 1) and the slow (reaction 2) P515 response, to flashes fired before, during and after a 20 sec period of continuous illumination, under optimal redox poising of the cyclic system. The flash responses immediately after the light periods reveal that reaction 1 is fully restored. However, the recovery of reaction 2 takes about 100 sec. Unfortunately, for technical reasons, no measurements of the ATP hydrolysing rate could be performed in this experiment. Identical results were found following an upward pH jump after preacidification of the vesicle lumen with succinic acid according to the classical Jagendorf and Uribe experiment (19). From Fig. 3.7 it can be seen that

during the relaxation of the transmembrane pH (in this case with a halftime of about 50 sec) the amplitude of reaction 2 is strongly reduced, while the amplitude of reaction 1 remains essentially unaffected.

3.4 Discussion

According to Petrack (20) illumination of chloroplasts with strong light in the presence of thiol reagents results in the activation of the latent ATPase. In our experiments, we demonstrate a partial need for artificial thiol reagents in suspensions of chloroplasts broken in the absence of Mg^{2+}. From Table I it can be seen that the chloroplast ATPase in intact chloroplasts can be fully activated by illumination alone. In accordance with Mills and McKinney (21,22) we suggest that activation of the chloroplast ATPase in intact chloroplasts is modulated by the physiological dithiol protein thioredoxin which is reduced by ferredoxin via Fd-thioredoxin reductase in the light. Recently it has been suggested (23) that the thiol modulator of the ATPase is loosely bound to the membrane in a MG^{2+}-dependent mode. In accordance with this we find that the chloroplast ATPase in chloroplasts, broken in the presence of 4 mmol/liter $MgCl_2$, can be activated by illumination in the absence of DTE. In our experiments excess amounts of ATP were present in the sample during the illumination period (see Table I). We suggest that under these conditions activation of the chloroplast ATPase still occurs by modulation of the CF1 part of the ATPase by the physiological thiol modulator. The efficiency of the system is somewhat less than in intact chloroplasts. This loss might be caused by a partial removal of intermediary compounds from the thylakoid membrane during the breaking procedure. Apparently the lower efficiency is partly compensated by the relatively high concentration of ATP. The addition of DTE to a suspension of chloroplasts, broken in the presence of 4 mmol/liter $MgCl_2$, did not further increase the rate of ATP hydrolysis (Table I). On the contrary, pronounced effects of the addition of DTE on the capacity to hydrolyze added amounts of ATP were found in chloroplasts, broken in the absence of $MgCl_2$. The ATPase present in these chloroplasts, which could not be activated by illumination alone, became activated by illumination in the presence of 1 mmol/liter DTE (data not shown).

From Figs. 3.3 and 3.4 and Table I, it can be seen that activation of the chloroplast ATPase coincides with a temporary suppression of the slow

component (reaction 2) of the P515 shift induced by a light flash. Under conditions in which the chloroplast ATPase is activated and ATP hydrolysis takes place, no reaction 2 can be measured, neither in intact nor in broken chloroplasts. This is in conformation with results reported by Schreiber and Rienits (11). Moreover, from Fig. 3.4 it can be seen that the duration of the suppression of reaction 2 after activation of the ATPase depends on the amount of ATP present in the sample. Further evidence that activation of the ATPase is involved in this phenomenon stems from the fact (see Fig. 3.5) that an inhibition of the ATPase by tentoxin results in prevention of the suppression of reaction 2. This seems true for both intact and broken chloroplasts. It was found that in the presence of tentoxin no ATP hydrolysis could be induced (Table I). Ample evidence has been provided that upon ATP hydrolysis reverse coupling reactions are induced (for a review, see (24). Reverse coupling has been demonstrated to be accompanied by the generation of a membrane potential, as suggested by an electrochromic response of P515 (11) and by the response of the extrinsic-field-indicating probe oxonol VI (25,26). There seems to be a strict complementarity of the ATP-induced and light-induced absorbance changes around 515 nm with respect to reaction 2 (11). These results, in combination with the results described in this communication, give strong support for the proposal (3) that the P515 bandshift is composed of two separate reactions called reaction 1 and reaction 2. In this interpretation reaction 1 is associated with a light-induced charge separation across the thylakoid membrane and a consequent single exponential dark decay of the created trans-membrane electric field, presumably by ion-charge diffusion. This reaction 1 type electrochromic shift is independent of a preestablished energetic state of the membrane created by either a preceding light flash or a period of ATP hydrolysis. On the contrary, the reaction 2 type electrochromic shift proves to be extremely sensitive toward a preestablished energetic state of the membrane. As shown in Fig. 3.1, reaction 2, i.e., the second slow rise, is largely absent in the P515 response of a single flash following two preceding flashes separated in time for 100 msec. Induction of ATP hydrolysis in the dark also causes a complete suppression of reaction 2 in a light flash during a period dependent on the amount of ATP present in the sample (Fig. 3.4). Also in subchloroplast vesicles, reaction 2 is absent from the signal under conditions at which the membrane is energized either by preillumination (Fig. 3.6) or by an artificially induced ΔpH (Fig. 3.7). In

accordance with Schapendonk and Vredenberg (3) and with Schreiber and Rienits (11), we suggest that reaction 2 finds its origin in intramembranal local fields in the vicinity of the P515 pigment complex. These fields obviously can be created either by light-driven electron transport or by ATP hydrolysis. The energetic requirement to generate these fields apparently is low, because of the small number of single saturating light flashes required to saturate reaction 2, and the long periods at which it can be sustained by an activated ATPase. A tentative model describing the origin of reaction 2 in terms of such local intramembranous fields has recently been published (27).

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CHAPTER 4, ONSET OF ATP SYNTHESIS IN SPINACH CHLOROPLASTS AFTER SINGLE TURNOVER FLASHES IN RELATION TO ADENYLATE KINASE ACTIVITY.

In collaboration with O. van Kooten and W.J. Vredenberg. To be published in FEBS Letters.

Summary

Flash-induced ATP synthesis was investigated in chloroplasts isolated from spinach varieties with a pronounced difference in their adenylate kinase activity. In chloroplasts with high adenylate kinase activity the first 4 flashes induced ATP hydrolysis which persisted for an extended dark period at which the membrane remained energized. In chloroplasts showing low adenylate kinase activity, ATP hydrolysis could only be induced by a successive flash train of at least 60 flashes or by a 30 to 60 sec period of continuous pre-illumination. The activity of the adenylate kinase must be considered when the kinetics of initial ATP synthesis and of P515 in light flashes are studied.

4.1 Introduction

Direct monitoring of flash-induced ATP synthesis in chloroplasts has been reported by Schreiber and Del Valle-Tascon (1). Using the firefly luciferase method both ATP synthesis and hydrolysis could be measured simultaneously in the same chloroplast suspension. These experiments showed that in fully dark-adapted chloroplasts the first 6 flashes induce ATP consumption and only after a certain period of ATP hydrolysis net ATP synthesis was observed. The onset of initial ATP consumption, following 8 hours of strict dark adaptation, could be shown to be triggered by as little as 2 single turnover saturating actinic flashes given 1 s apart (1). Accordingly, Graan and Ort (2) reported flash-induced ATP synthesis in chloroplasts using a sensitive P^{32} method. In agreement with Schreiber and Del Valle-Tascon, these authors found flash-induced ATP yields provided 5 activating flashes were given. Unfortunately, their method did not allow to monitor ATP synthesis parallel to ATP hydrolysis. However, ATP hydrolysis has been shown to occur in intact and freshly broken chloroplasts and has been reported to pertain for extended periods of darkness after light activation of the reversible ATPase (3-6, chapter 3). The activated membrane-bound ATPase is known to invoke a considerable electrochemical potential gradient of protons ($\Delta\mu H$)(7-12). This gradient that is present in the dark as a result of ATP hydrolysis can be considered as a "threshold" energy for ATP synthesis. Consequently, even small increments of the proton motive force could result in the formation of ATP under these conditions.

This may explain the rate of ATP synthesis found in (1) which increased with the number of flashes to a quasi-stationary value of 50 nmole ATP.mg^{-1}Chl.min^{-1} and equivalent to about 1 ATP per CF1 per flash. The same optimal yield of flash-induced ATP synthesis was found in (2).
The effect of the first activating flashes on the reversible ATPase will be dependent on the value of ΔG_{ATP} which is affected by adenylate kinase activity. When there is a high adenylate kinase activity, as for instance in the experiments reported in ref. 1, addition of ADP will result in the conversion of a significant amount of ADP into ATP in the dark. This dark conversion will clearly affect ΔG_{ATP} and will favour the conditions for ATP hydrolysis by the reversible ATPase.

In order to study the effect of the first activating flashes on the reversible ATPase in dark-adapted chloroplasts in relation to the adenylate kinase activity, we compared these effects for two types of chloroplasts which showed a pronounced difference in the endogeneous adenylate kinase activity. It is shown that in spinach chloroplasts of a summer variety with a high adenylate kinase activity the first 4 flashes result in ATP hydrolysis. In chloroplasts of a winter variety with a low activity of the enzyme at least 60 flashes are needed. In both types of chloroplasts, flash-induced ATP synthesis was about 1 molecule ATP per CF1 per flash provided the ATPase was pre-activated. The energy state of the thylakoid membrane after activation of the ATP hydrolase was reflected in the kinetics of the flash-induced P515 electrochromic bandshift as was reported in chapter 3. These experiments demonstrate that the length of the pre-illumination period (i.e. the number of activating flashes) needed to activate the reversible ATPase is dependent on the value of ΔG_{ATP}. This potential is affected by the activity of the adenylate kinase enzyme. A high activity of the enzyme will result in conditions that are favourable for ATP hydrolysis by the membrane bound ATPase. Since this hydrolysing activity of the ATPase will result in energization of the membrane, and in this way contribute to the energetic state which determines light-driven ATP production, the adenylate kinase activity must be taken into account when studies of flash-induced ATP synthesis and of P515 kinetics are performed.

4.2 Materials and methods

Freshly grown spinach (Spinacia oleracea) was used for all experi-

ments. The plants were grown in a greenhouse under high-pressure mercury lamps (Philips MGR 102-400) at an intensity of approximately 100 $W.m^{-2}$ with a light period of 8 hrs per day. Provisions were made to keep the temperature at the leaf and soil surface at 18 to 20 °C. The relative humidity of the atmosphere was minimal 70 per cent. Two different spinach cultivars were used. One cultivar (cv Nobel), known as a summer variety, has been found to require a light intensity of at least 200 $W.m^{-2}$ for optimal growth. The other cultivar (cv Bergola), a winter variety, grows optimally at a light intensity of about 80 $W.m^{-2}$. Both types of plants were grown under identical environmental conditions. The procedures used for the isolation of chloroplasts were also the same. Intact chloroplasts routinely were isolated according to a modified method of (13) as described in chapter 2. This procedure yielded preparations with 90 to 95 per cent intact chloroplasts as determined by ferricyanide reduction (14). Following isolation, chloroplasts were stored in the dark at 0 °C at a chlorophyll concentration of approximately 1.0 mg/ml. Shortly before each experiment 60 µl chloroplasts were osmotically ruptured by mixing with 1 ml hypotonic medium consisting of Hepes-KOH 5 mM (pH 7.5), Mg-acetate 5 mM and DTE 4 mM. After a 60 sec incubation the suspension was made half isotonic by the addion of 1 ml of the following buffer; sorbitol 330 mM, Hepes-KOH 20 mM (pH 7.5), NaH_2PO_4 2 mM, Mg-acetate 2 mM and DTE 2 mM. Final chlorophyll concentration was 30 µg/ml.

The chloroplast suspension was continuously stirred in a 3 ml cuvette thermostated at 10 °C. ATP concentration changes were measured with the luciferin-luciferase luminescence assay in an appropriate monitoring device (15), equiped with a photomultiplier. For each experiment 200 µl of a solution of "ATP monitoring reagent" (LKB Wallac, the vial content being dissolved in 10 ml double-distilled water) was added to the cuvette. ADP (Boehringer) was added at a concentration of 20 µM. ATP calibration was done with known amounts of a freshly prepared 10 µM ATP solution (LKB-Wallac). Saturating single-turnover actinic flashes with a half-width of 8 µs were transmitted to the sample via light guides. Pre-illumination with red light came from a 250-W tungsten lamp and was transmitted to the sample via light guides. The photomultiplier was shielded from actinic ligth by an appropriate filter combination. Absorbance changes at 518 nm, induced by single-turmover flashes, were measured in a modified Aminco-Chance absorption difference spectrophotometer as described in chapter 2.

4.3 Results and Discussion

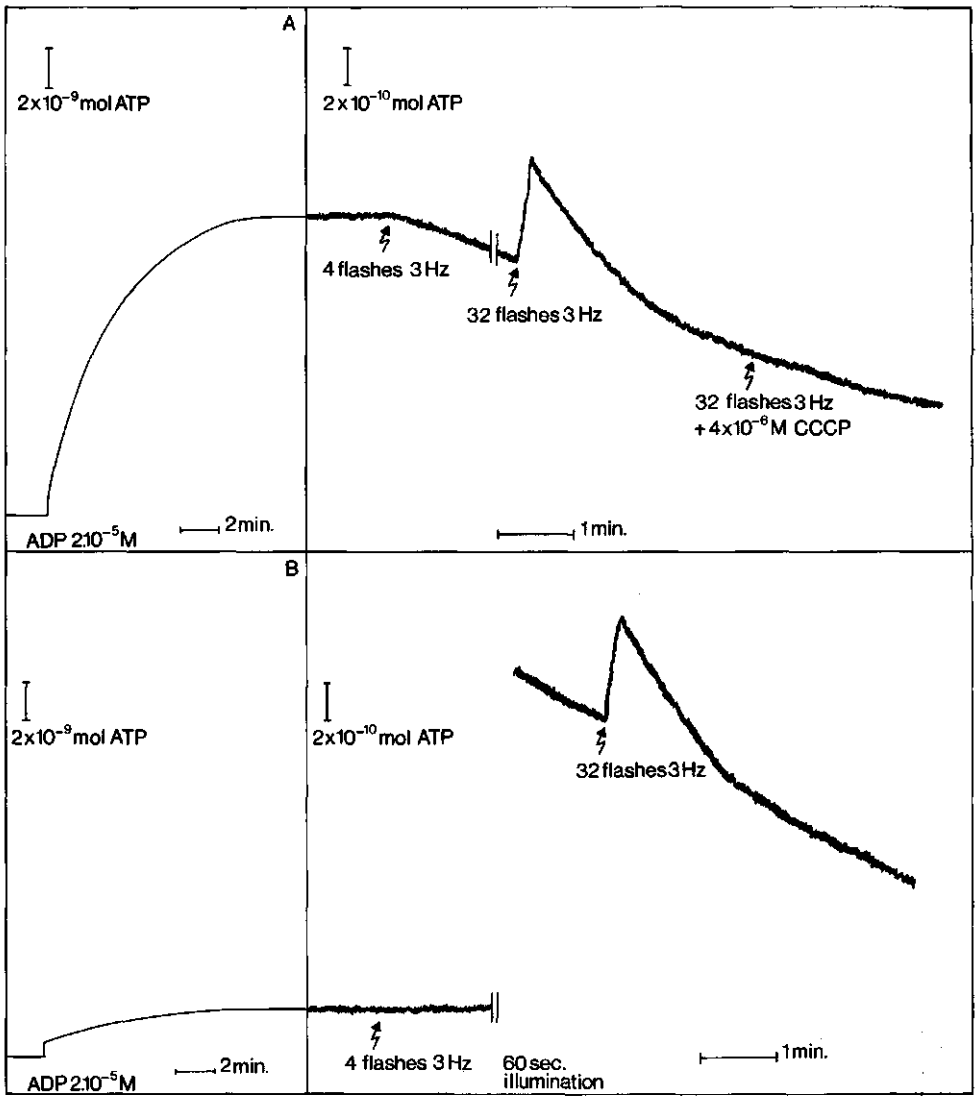

Fig. 4.1. Monitoring of ATP with the firefly luciferase method in chloroplasts isolated from a summer (4.1A) and a winter (4.1B) spinach variety. **Lefthand-side:** response due to adenylate kinase activity upon the addition of 2 X 10^{-5} M ADP. **Righthand-side:** luminescence response following illumination of chloroplasts with successive flash trains of 4 and 32 flashes, determined on a 10 times more sensitive scale.

Left hand part of figs. 4.1A and 4.1B shows the luciferine luminescence response upon the addition of 20 μM ADP to samples of dark-adapted chloroplasts isolated from the summer (4.1A) and the winter (4.1B) spinach variety, respectively. A biphasic rise of luminescence upon addition of ADP is obvious in both samples. The rapid phase (about equal in both samples) is due to contaminating ATP (0.1 %) in the ADP solution. The slow phase is due to the action of adenylate kinase. There appears to be a pronounced difference with respect to the enzyme activity between the two chloroplast preparations. This activity can be calculated using the second-order rate constant of the enzymic reaction and the amount of ADP converted. In summer chloroplasts, at the concentration of ADP used, the data of fig. 4.1A yield a rate constant of about 144 $M^{-1}.s^{-1}$. From fig. 4.1A it can be seen that in the summer spinach variety about 50 per cent of the the amount of added ADP has been converted into ATP in the dark due to this activity. Approximately the same kinase activity and rate of ADP conversion can be calculated from the data in fig. 1 in reference 1. Under exactly the same experimental conditions, the activity of the adenylate kinase enzyme in winter chloroplasts can be calculated to be about 8.4 $M^{-1}.s^{-1}$ (fig. 4.1B). In these chloroplasts, the amount of ADP converted into ATP in the dark was found to be much less (maximal 10 per cent, fig. 4.1B).

This conversion of added ADP into ATP prior to illumination will affect ΔG_{ATP}, favouring ATP hydrolysing conditions. As a result, conditions in favour of ATP hydrolysis will be more pronounced in the sample of chloroplasts isolated from summer type spinach. After the kinase-dependent dark reaction was completed, 4 saturating flashes were given at 3 Hz repetition rate every 4 minutes and the luminescence signal was monitored on a 10 times more sensitive scale. The right hand side of fig. 4.1A shows that in summer chloroplasts the first series of four flashes induce ATP hydrolysis in the subsequent dark period. In accordance with results of others (1) and our results in chapter 3, it was found that this hydrolysis of ATP continued for an extended dark period.As was reported in chapter 3, ATP hydrolysis in the dark results in energization of the thylakoid membrane. This energization has been shown to be reflected by altered kinetics of the flash-induced P515 electrochromic bandshift. The slow-phase (i.e. reaction 2), contributing to the overall signal in dark-adapted chloroplasts is virtually absent under energized conditions of the membrane. From fig.4.2A it can be seen that in summer chloroplasts after 4 activating flashes, the

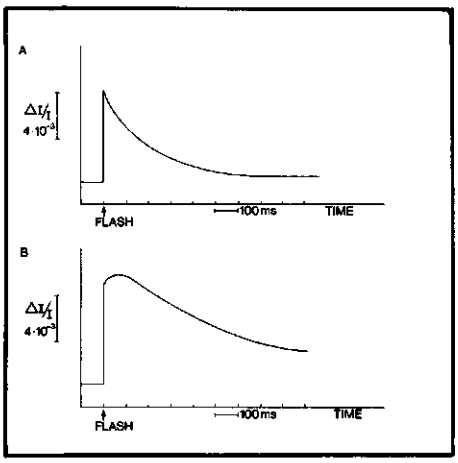

Fig. 4.2. Flash-induced absorbance change at 518 nm in chloroplasts determined after illumination with 4 successive flashes (repetition rate 3 Hz) in chloroplasts isolated from a summer type spinach (A) and from a winter type (B).

kinetics of the flash-induced P515 bandshift indeed is devoid of the slow-phase normally present in dark-adapted membranes. A second flash train (32 flashes), given 4 minutes after the first 4 activating flashes caused a net synthesis of ATP (fig. 4.1A), In this experiment the ATP yield per flash was about 1 nmol ATP. mg chl^{-1}.Assuming a photosynthetic unit size of 500 chl molecules this means 0.5 molecule ATP is formed in every electron transport chain per flash. Considering that there is 1 CF1 for every 1000 chlorophyll molecules this means that with each flash 1 ATP molecule is formed for every CF1 which means optimal ATP formation. Since the flash-induced luminescence response was found to be completely abolished by the addition of 4 μM CCCP (fig. 4.1A) the luminescence change undoubtedly reflects ATP formation.

From fig. 4.1B (right hand side) it can be seen that in winter chloroplasts, where adenylate kinase activty is much lower than in summer chloroplasts, the first 4 activiting flashes did not cause a change in the luminescence signal, i.e. a change in ATP concentration. The same was found for 0.05 Hz repetitive illumination with series of four activating flashes (data not shown). ATP hydrolysis in these chloroplasts could only be induced by a series of at least 60 flashes or by a 30 to 60 second period of continuous pre-illumination. Once hydrolysis was induced, flash-induced ATP synthesis could be measured. Under these conditions the yield of ATP per flash was equal to the yield reported for summer chloroplasts (fig. 4.1B). From fig. 4.2B it can be seen that the kinetics of the flash-induced P515 bandshift determined in winter chloroplasts after one series of 4 ac-

tivating flashes is similar to the kinetics normally found in dark-adapted chloroplasts, i.e. shows a pronounced contribution of the slow-phase. This result is in agreement with the observation that in these chloroplasts the ATPase cannot be activated by short periods of illumination.

These experiments show that the light energy requirement for the activation of the chloroplast ATPase is different in samples that show a difference in adenylate kinase activity. High activity of the enzyme, as was found in our summer type chloroplasts,results in conditions in which ATP hydrolysis could be provoked by as little as 4 activating flashes. Chloroplasts with a low activity of the enzyme require substantially longer periods of illumination for the activation of ATPase. Activation of the ATPase resulted in dark ATP hydrolysis and sustained energization of the membrane in both samples. The latter is indicated by a pronounced alteration in the kinetics of the flash-induced P515 electrochromic bandshift. Under pre-energized conditions flash-induced ATP synthesis was found to be similar in both samples and reached an optimal value of about 1 molecule of ATP formed per flash and CF1. These results strongly suggest that factors determining ΔG_{ATP} in the dark (e.g. adenylate kinase activity) must be taken into account when kinetic studies of flash-induced ATP synthesis are made. This has insufficiently been considered in the past. The data illustrate the usefulness of the flash-induced P515 response as a qualifying monitoring probe for the energy requirement and maintenance of the activity of the membrane ATPase.

The procedure so far followed for the determination of the P515 response in general includes signal averageing, in order to obtain a sufficient signal to noise ratio. It is likely that in plant material with a high adenylate kinase activity the first flashes of a series used for the averageing procedure result in activation of the chloroplast ATPase. The subsequent hydrolysing activity of the ATPase will result in energization of the thylakoid membrane and as a consequence, the P515 response in subsequent flahses is devoid of the slow-phase. Consequently the averaged P515 signal then does hardly, if at all, show a contribution of the slow phase (Reaction 2). These results explain the fact that in the summer spinach variety grown under sub-optimal light conditions (for instance with lower light fluence in winter time) the flash-induced 515 nm electrochromic bandshift determined in intact leaves is found to be devoid of the slow-phase. This absence of the slow-phase in the P515 signal is a rather common

seasonal phenomenon, which has plagued our experiments for a long time. It remains to be elucidated whether the adenylate kinase activity in the plant leaves is dependent on the light conditions at which the plants are grown.

With improved measuring techniques one is now able to reach a good definition of the P515 response with a single flash activation. This enables to discriminate whether or not the absence of the slow-phase in the P515 signal is due to altered membrane constitution (16), or to sustained membrane energization.

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CHAPTER 5, THE KINETICS OF THE FLASH-INDUCED P515 RESPONSE IN RELATION TO THE H^+-PERMEABILITY OF THE MEMBRANE BOUND ATPASE IN SPINACH CHLOROPLASTS.

In collaboration with O. van Kooten and W.J. Vredenberg. Modified version of publication in Journal of Bioenergetics and Biomembranes, Vol. 17, No. 4 (1985) 207-216.

Abstract

The effect of dicyclohexylcarbodiimide (DCCD) on the kinetics of the flash-induced P515 response and on the activity of the ATPase was investigated in isolated spinach chloroplasts. It was found that after the addition of 5 x 10^{-8} mol DCCD the rate of ATP hydrolysis induced by a period of 60 sec illumination was decreased to less than 5% of its original value. At this concentration, hardly any effect, if at all, could be detected on the kinetics of the flash-induced P515 response, neither in dark-adapted nor in light-activated chloroplasts. It was concluded that the presence of concentrations of DCCD, sufficiently high to affect the ATPase activity, does not affect the kinetics of the flash-induced P515 response. Since DCCD decreases the H^+ permeability of the membrane-bound ATPase, it was concluded that this permeability coefficient for protons is not an important factor in the regulation of the flash-induced membrane potential and, therefore, does not affect the kinetics of the flash-induced P515 response.

5.1 Introduction

Ample evidence has been presented, both in chloroplasts and in green algae, that the overall decay rate of the flash-induced P515 response is substantially accelerated after short periods of illumination (1-3; chapter 3 and chapter 4 of this thesis). It has been concluded, following the interpretation of others (4.5), that the decay rate is mainly determined by the H^+ efflux through the ATPase, i.e., is higher under conditions at which the chloroplast ATPase is activated. In accordance with this, Morita (6) demonstrated a seeming connection between the activity of the ATPase and the apparent P515 decay rate. On the other hand, according to an analysis by Schapendonk (7), there is some doubt as to whether protons contribute significantly to the membrane conductance, even under phosphorylating conditions. According to Schapendonk (8), the decay of the P515 absorbance change in chloroplasts is almost exclusively determined by the decay of a special type of electrochromic change (called reaction 2), which is not directly linked to a transmembrane potential but rather to an intramembranal, local field in the vicinity of the P515 pigment complex. In our present interpretation, the reaction 2 component of the P515 response is the reflection of an intramembranal electrical phenomenon, presumably

associated with the liberation of protons in inner-membrane domains near the Fe-S cyt b-f protein complex (9,10).

It has been suggested (11) that these domains are connected via lateral H^+-conductive channels with other membrane domains that act as proton sinks (i.e., the ATPase). In this respect it is of interest to mention that, in conformation with the results of others (12,13), we have shown that reaction 2 can also be induced in the dark toward its saturation level by ATP-driven electron flow (proton translocation) (see chapter 3 of this thesis).

It was found that the contribution of the reaction 2 component to the P515 response, which is most obvious in dark-adapted membranes, appears to be fully suppressed after short periods of illumination, i.e., under conditions in which the chloroplast ATPase is activated. This suppression of reaction 2 was shown to be temporary. The length of the dark period following (pre)illumination which is needed for a full recovery of reaction 2 appeared to be dependent on the amount of ATP present in the sample and could be correlated with the length of the period during which ATP hydrolysis took place in the dark.

Obviously, the acceleration of the overall decay rate of the flash-induced P515 response found after short periods of illumination can also be explained by the suppression of the reaction 2 component. Therefore, this acceleration is not necessarily linked to an enhancement of proton efflux via the chloroplast ATPase.

In order to test this alternative explanation, we have investigated the effect of the energy transfer inhibitor compound DCCD on the kinetics of the flash-induced P515 response and on the activity of the ATPase. It is concluded that concentrations of DCCD, sufficiently high to affect the ATPase activity, have no effect on the kinetics of the P515 response either in dark-adapted or in light-activated chloroplasts. Obviously, the reduction of the H^+ permeability of the thylakoid membrane-bound ATPase caused by DCCD has no effect on the kinetics of the P515 response. Therefore, we conclude that this permeability for protons is not an important factor in the regulation of the flash-induced membrane potential and does not affect the decay rate of the P515 response induced by a single turnover saturating light flash.

5.2 Materials and Methods

Freshly grown spinach (Spinacia oleracea cv: Bergola) was used for all experiments. The plants were grown under high-pressure mercury lamps (Philips MGR 102-400) at an intensity of approximately 100 W m^{-2} with a light period of 8 hours per day. Provisions were made to keep the temperature at the leaf and soil surface at 18-20°C. The relative humidity of the atmosphere was minimal (70%).

Intact chloroplasts were routinely isolated according to a modified method of Walker (14) as described in chapter 2. This procedure routinely yielded preparations with 90-95% intact chloroplasts as determined by ferricyanide reduction (15). Broken chloroplasts were obtained by a 60-sec osmotic shock on ice, in a medium containing 5 mmol/liter MgAc and 10 mmol/liter Tricine adjusted to pH 7.8 with NaOH, and subsequent addition of double strength assay medium. The final composition of the assay medium is indicated in the legends to the figures.

Absorbance changes at 518nm induced by single turnover saturating light flashes in isolated chloroplasts were measured in a modified Aminco Chance absorption difference spectrophotometer as described in chapter 2. Preillumination with red light came from a 250-W tungsten lamp and was transmitted to the sample via light guides and appropriate filters.

ATP hydrolysis in chloroplasts was determined with the bioluminescent firefly luciferine-luciferase assay method as described in chapter 3.

All measurements were performed at 10° C. DCCD was added to the sample, prior to the illumination period, from stock solutions containing 96% ethanol. The ethanol concentration in the reaction medium never exceeded 2%.

5.3 Results and Interpretation

A representative example of the time course of the absorbance change at 518 nm (ΔA518) upon a single turnover light flash in dark-adapted, broken chloroplasts is illustrated in Fig. 5.1A.

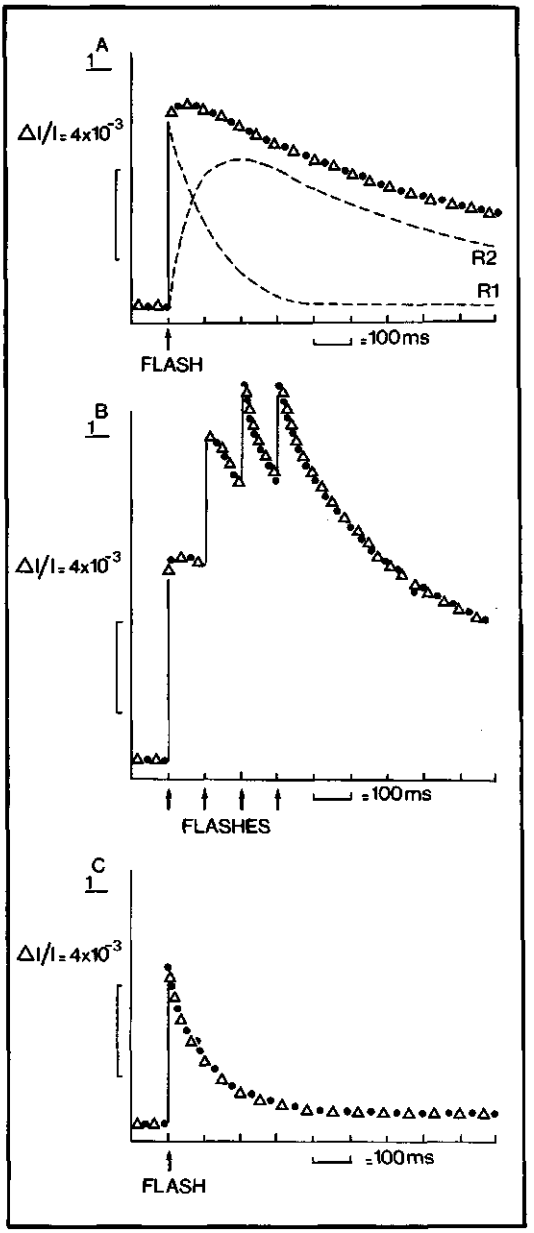

Fig. 5.1. Absorbance changes at 518 nm in dark-adapted broken chloroplasts induced by a single flash (A), and by a series of four successive flashes fired at 100-msec time intervals (B) measured in the absence (●) and presence (Δ) of 5 x 10^{-8} mol DCCD. (C) Absorbance change induced by a single flash after a period of 60-sec illumination, in the presence (Δ) and absence (●) of 5 x 10^{-8} mol DCCD. All measurements were performed in a medium containing chlorophyll 75 µg/3ml, Hepes-KOH 20 mM (pH 7.5), sorbitol 330 mM, MgAc 2mM, KH_2PO_4 2mM, DTE 2 mM, and ATP 10^{-5}M.

From this figure it can be seen that $\Delta A518$ under these conditions occurs with multiphasic rise and decay kinetics. By using double flashes it has been shown (7; chapter 3) that the single flash response curve can be deconvoluted into at least two separate responses. These reactions (i.e., reactions 1 and 2), determined in our experiments according to the aforementioned procedure, are indicated in the figure by the dashed curves.

In our present interpretation (9,10), reaction 1, characterized by a fast rise and a single exponential dark decay, is the reflection of the generation and decay, respectively, of a transmembrane delocalized electric field induced by the light-induced charge separation in PSI and PSII. Reaction 2, characterized by a relative slow increase in absorbance after the flash and a relatively slow decay with a first-order rate constant, which is severalfold the rate constant of the decay of reaction 1, is related to intramembranal electrical phenomena (8,10,12,13) presumably associated with the liberation and subsequent stabilization of protons in innermembrane domains near the cyt b-f protein complex (9). As can be seen from Fig. 5.1A, the decay rate of the overall P515 response in dark-adapted chloroplasts is almost exclusively determined by the decay of reaction 2. As was shown in chapter 3, reaction 2 is largely reduced or even absent from the P515 response under conditions at which the thylakoid membrane is pre-energized. In the Figs. 5.1B and 5.1C, respectively, this is shown for the situation at which energization is brought about either by light-driven electron transport (proton translocation) or by reverse electron transport (proton translocation) caused by ATP hydrolysis in the dark. From Fig. 5.1B it can be seen that the contribution of reaction 2 to the P515 response (i.e., the second slow rise), most obvious in the first flash, decreases significantly after two or more following flashes separated in time by 100 ms. This reduction in the contribution of the reaction 2 component of the P515 response was found to be temporary and could be completely overcome by a period of 10 sec dark adaptation (data not shown). Figure 5.1C shows the effect of a period of 60 sec illumination of broken chloroplasts on the kinetics of the P515 response. As can be seen, the reaction 2 component of the response induced by a light flash given 5 sec after the illumination period is completely suppressed. This suppression of reaction 2 caused by preillumination could only be found when ATP and DTE were present during the illumination period, i.e., under conditions at which the chloroplast ATPase was activated. Also in this

case, the suppression of reaction 2 was found to be temporary; however, the duration of the suppression period was much longer (30 min). The length of the suppression period appeared to be dependent on the amount of ATP present, and could be well correlated with the time ATP hydrolysis could be detected, as was shown before in chapter 3.

Obviously, the acceleration of the overall decay rate of the P515 response generally found after short periods of illumination of chloroplasts (2,3) and green algae (1) can be explained by the suppression of the reaction 2 component therein and, therefore, is not necessarily linked to an enhancement of proton efflux via the membrane-bound ATPase as suggested by others. In order to test this alternative explanation, the effect of the energy transfer inhibitor compound DCCD on the kinetics of the flash-induced P515 response and on the activity of the ATPase was investigated. DCCD was added in our experiments up to a concentration of 10^{-7} mol which means a concentration ratio of DCCD to ATPase molecules of approximately 1000:1. From Fig.5.2 it can be seen that the activity of the ATPase induced by a 60-sec period of illumination of a sample of broken chloroplasts in the presence of DTE and ATP is decreases from 40 µmol ATP/mg chl./hr in the absence of DCCD to 1.4 µmol ATP/mg chl./hr in the presence of 5 x 10^{-8} mol DCCD.

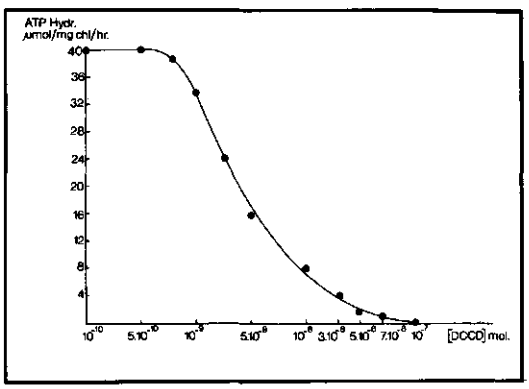

Fig. 5.2. Rate of ATP hydrolysis measured in broken chloroplasts after a period of 60-sec illumination as a function of the concentration of DCCD. The measurement was performed in medium (volume 3 ml) containing chlorophyll 75 µm/3ml, Hepes-KOH 20 mM (pH 7.5), sorbitol 330 mM, MgAc 2 mM, KH_2PO_4 2mM, DTE 2 mM, and ATP 10^{-5}M.

At this concentration, no significant effect could be detected on the kinetics of the P515 response induced by a single flash in dark-adapted chloroplasts (see Fig. 5.1A). Obviously, at this low concentration DCCD

does not cause inhibition of electron transfer as reported by Uribe (16) for higher concentrations. As can be seen from Fig. 5.1B, the P515 response induced by a series of four following light flashes fired at 100-msec time intervals is identical, both in the presence and absence of DCCD. In both cases, the P515 response curve could be characterized by the specific loss of the reaction 2 component after the second flash. The acceleration of the decay rate of the response found after the third flash appeared to be identical both in the control and in DCCD-treated chloroplasts. Obviously, these observed alterations in the kinetics of the flash-induced P515 response cannot be explained by the enhancement of proton efflux from the thylakoid mediated by the activation of the chloroplast ATPase. If this were true, the presence of 5×10^{-8} mol DCCD should have prevented these alterations to occur. Moreover, if the ultimate decay rate of the P515 response after the third flash is regulated by the proton flux through the membrane-bound ATPase, then it would be expected to be lower in the presence of DCCD. The same results, qualitatively, were found when chloroplasts were preilluminated for a period of 60 sec in the presence of DTE and ATP (see Fig. 5.1C). From this figure it can be seen that the P515 response induced by a light flash given 5 sec after the illumination period can be characterized by a fast rise and a subsequent single exponential dark decay with a first-order rate constant of about 10 sec^{-1} (i.e., reaction 1). Also in this experiment, the presence of 5×10^{-8} mol DCCD, which

Number of mols DCCD added to the sample.	------	5×10^{-9}	5×10^{-8}
Rate of ATP hydrolysis μmol ATP/mg chl./hr.	40	13	1,4
Time period (min) ATP hydrolysis could be detected.	30	12.	1,5

Table I. Rate of ATP Hydrolysis and the Length of the Period that ATP Hydrolysis could be Detected in Broken Chloroplasts after 60-sec Illumination in the Presence and Absence of DCCD.*

*The measurement was performed in a medium as described in the legend of Fig. 1.

is shown to be a very effective concentration for the inhibition of the ATPase (see Fg. 5.2), did not influence the effect of illumination on the P515 response and, moreover, had no effect on the ultimate decay rate.

From these experiments we conclude that reduction of the H^+ permeabi-

lity of the thylakoid membrane-bound ATPase caused by DCCD has no effect on the kinetics of the P515 response induced by a light flash. Accordingly, the suggestion made by others (1,2,3,4,5) that changes in the proton flux due to ATPase activation might affect the overall kinetics of the P515 response does not seem plausible. The observed acceleration of the overall decay rate of the P515 response can be explained by the suppression of the reaction 2 component of the response which takes place under conditions where the thylakoid membrane is energized, either by light-driven electron transport or by reverse electronflow caused by ATP hydrolysis in the dark. Even in the presence of 5 x 10^{-8} mol DCCD, the more than 95% reduced activity of the ATPase could account for the complete suppression of reaction 2; however, the duration of the suppression period appeared to be significantly reduced. As can be seen from Fig. 5.3, the suppression of reaction 2 by a period of 60 sec illumination of broken chloroplasts in the presence of DTE and ATP is diminished from 30 min in control chloroplasts to 2 min in the presence of 5 x 10^{-8} mol DCCD. This decreased duration of the suppression period in the presence of DCCD could be well correlated with the decreased period ATP hydrolysis could be detected (see Table I).

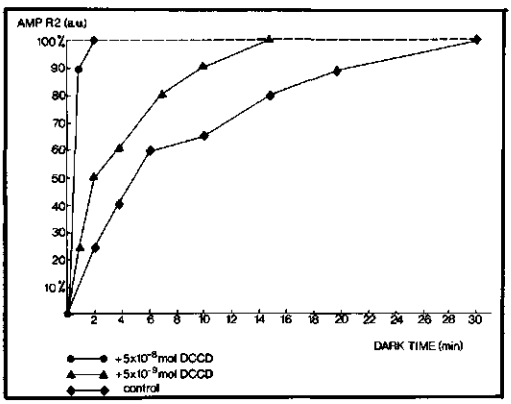

Fig. 5.3. The effect of dark adaptation after a 60-sec preillumination on the recovery of the amplitude of reaction 2 in chloroplasts in the absence (♦) and presence of 5 x 10^{-9} (▲), or 5 x 10^{-8}mol DCCD (●). The measurement was performed in a medium containing chlorophyl 75 µg/3 ml, Hepes-KOH 20 mM (pH 7.5), sorbitol 330 mM, MgAc 2mM, DTE 2 mM, KH_2PO_4 2mM, and ATP 10^{-5}M.

This finding is important for the interpretation of the experimental results reported by Morita et al. (6). In these experiments it was shown that the acceleration of the P515 response in spinach leaves, normally found after short periods of illumination, was prevented by the addition of 1 mM DCCD. This result, which appears to be in conflict with our results in

chloroplasts, seems to support the suggestion that the decay rate of the P515 response is determined by the H^+ efflux through the ATPase. However, in Morita's experiments, the flash-induced absorbance change was measured 90-170 s after the illumination period. From Fig. 5.3 it can be seen that in the presence of DCCD, at least in chloroplasts, the suppression of reaction 2 was sustained for a maximum period of 90 sec. This means that after 90 sec dark adaptation, the P515 response induced by a light flash was found to be identical to the response obtained before the illumination period. Obviously, the results reported by Morita (6) can be explained by the relative long period of dark adaptation preceding the actinic light flash.

From these experiments we conclude that the reduction of the H^+ permeability of the thylakoid membrane caused by DCCD has no effect on the kinetics of the flash-induced P515 response. Therefore, this permeability for protons is not an important factor in the regulation of flash-produced membrane potential and does not affect the decay rate of the flash-induced P515 response. The acceleration of the P515 response generally found after short periods of illumination can be explained by the selective suppression of the reaction 2 component of the P515 response, caused by reverse electron flow (cf. proton translocation) in the dark, following light activation of the chloroplast ATPase.

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CHAPTER 6, THE KINETICS OF P515 IN RELATION TO THE LIPID COMPOSITION OF THE THYLAKOID MEMBRANE

In collaboration with O. van Kooten and W.J. Vredenberg. Modified version of publication in Journal of Bioenergetics and Biomembranes, Vol. 16, No. 4 (1984) 283-294.

Abstract

Flash-induced P515 absorbance changes have been studied in dark-adapted chloroplasts isolated from spinach plants grown under two different light intensities. The slow component (reaction 2), normally present in the P515 response of chloroplasts isolated from plants grown at an intensity of 60 $W.m^{-2}$, was largely reduced in chloroplasts isolated from plants grown at an intensity of 6 $W.m^{-2}$. This reduction of the slow component in the P515 response appeared to be coincident with an alteration in the lipid composition of the thylakoid membrane. Mainly the ratio monogalactosyldiacylglycerol to digalactosyldiacylglycerol appeared to be altered. In thylakoids from plants grown at 6 $W.m^{-2}$, the ratio was approximately 35% lower than that of plants grown at 60 $W.m^{-2}$. The amount of both cytochrome b_{563} and cytochrome f was largely reduced in chloroplasts isolated from plants grown at low light intensity. These results may indicate a possible correlation between structural organization of the thylakoid membrane and the kinetics of the flash-induced P515 response.

6.1 Introduction

It has been suggested by van Kooten (1) that the slow P515 response (i.e. reaction 2) is caused by a lateral and transverse delocalization of inner membrane electric fields associated with the liberation of protons in inner-membrane domains near the Fe-S cyt b-f protein complex. These domains might be connected via lateral H-conductive channels with other membrane domains which might act as proton sinks, i.e., ATP synthetase. Models depicting site-specific intramembranal proton processing in the thylakoid have been suggested by Dilley and by Kell (2,3). However, the existence of the proposed intramembrane structures capable of stabilizing protons within a hydrophobic matrix remains to be demonstrated.

In contrast to reaction 1, the occurrence of reaction 2 appears to be dependent on the functional integrity of the membrane, i.e., it disappears upon ageing, after a temperature shock and, as is shown in this paper, is largely reduced in plants grown at low light intensities. This paper deals with the question whether or not the occurrence and magnitude of reaction 2 in chloroplasts is associated with changes in the lipid composition of the thylakoid membrane. A comparative study has been made of light intensity

effects during plant growth on the kinetics of the flash-induced P515 response in isolated chloroplasts and on the lipid composition of the thylakoid membrane. We find that a significant reduction of reaction 2 in the P515 response observed in chloroplasts isolated from plants grown at low light intensity (less than 10 $W.m^{-2}$) is coincident with a 35% reduced ratio of the two major membrane lipids, MGDG to DGDG. The reduced ratio is mainly due to a reduced amount of MGDG in the low-light chloroplasts. In addition, the amount of both cytochrome b_{563} and cytochrome f in chloroplasts isolated from plants grown at 6 $W.m^{-2}$ is approximately 40% of the amount found in chloroplasts from plants grown at 60 $W.m^{-2}$. The concentration of other components of the electron-transport chain connecting photosystem 1 and photosystem 2 is approximately the same for both types of chloroplasts. This is qualitatively conclusive with results obtained with other plant species (4). The results presented in this paper may indicate a possible correlation between factors determining the structural organization of the thylakoid membrane and the kinetics of the flash-induced P515 response.

6.2 Materials and Methods

Spinacia oleracea (cv: Bergola) plants were grown in a growth chamber at 18° C and at 80% relative humidity. Plants were exposed to a daily light period of 8 hr at two different light intensities, 6 and 60 $W.m^{-2}$, respectively. Plants were harvested at the age of 7 weeks.

Intact chloroplasts were isolated according to a modified method of Cockburn (5) as described in chapter 2. This procedure routinely yielded preparations with 90-95% intact chloroplasts as determined by ferricyanide reduction (6). Broken chloroplasts were obtained by an osmotic shock on ice during 60 s, as described in chapter 3. Determination of Hill reaction rate and absorbance changes at 518 nm was performed as described in chapter 3.

Determination of total chlorophyll content and quantitative analysis of chlorophylls a and b were performed as described by Bruinsma (7). Spectral measurements were done with an Aminco DW-2A spectrophotometer. P_{700} was determined from light-induced absorbance changes at 700-740 nm, assuming an Σ of 64 $mM.cm^{-1}$ (8). Cytochrome f was estimated from the hydroquinone-reduced minus ferricyanide-oxidized difference spectrum in a medium containing Hepes-KOH 100mM (pH 7.0), Triton-X-100 (1% w/v), $MgCl_2$ 15 mM,

and EDTA 4 mM, using an Σ of 22 $mM.cm^{-1}$ (9). Cytochrome b_{563} was estimated from the dithionite-reduced minus ferricyanide-oxidized difference spectrum. An Σ of 20 $mM.cm^{-1}$ was assumed according to Rich (10). Reaction conditions were the same as for the determination of cyt f. Data analysis was performed as described elsewhere (11). The size of the PQ pool was estimated by comparing the fluorescence induction curves in the presence and absence of DCMU as described by Malkin (12).

For the determination of the lipid composition of the thylakoid membrane, envelope-free thylakoids (i.e., stripped thylakoids) were prepared by an osmotic shock during 60 s on ice in a medium containing Tricine-KOH 10 mM (pH 7.8), $MgCl_2$ 2mM, and KCl 2.5 mM. Stripped thylakoids were separated from the outer envelope fraction and a remnant of intact chloroplasts by sucrose gradient centrifugation according to Douce (13). Centrifugation was for 15 min at 2000 x g. Stripped thylakoids were recovered as a broad band at the interface of the 25 and 34% (w/w) sucrose layers. This band was aspirated, washed twice by centrifugation for 7 min at 4000 x g in a medium containing Hepes-KOH 50 mM (pH 7.5), sorbitol 330 mM, and $MgCl_2$ 2mM, and stored at 0° C. Lipids were extracted with ice-cold chloroform-methanol (1:2 by vol.) according to the procedure of Allen (14), except that the chloroform-methanol extract was washed with 0.1 M KCl instead of water. Lipids were separated on precoated TLC plates Silica Gel G-60 (20 x 20 cm, thickness 0.25 mm, 5 mg lipid applied) with acetone: benzene:water, 91:30:8 (v/v), as a developing agent (15). The plates were stained with rhodamine 6g (0.003% in a 4% NaOH solution) and immediately viewed under UV light at 366 nm. Glycolipids were stained on replicate plates with a solution of 0.5 g -naphthol in methanolwater 1:1 (v/v), according to Siakotos (16). Saponification of the lipids and methylation of the fatty acids with BF_3 in methanol were performed as described by Kuiper (17). Separation of the fatty acid methyl esters by gas-liquid chromatography and their identification was done by using a Becker gas chromatograph model 421 equipped with a flameionization detector. A 160-cm glass column with a diameter of 2 mm was used, filled with Gaschrom Q (100-120 mesh) coated with 5% Silicar 10-C. Nitrogen was used as carrier gas (60 $ml.min^{-1}$). Operating temperature was 130-170° C. The gas chromatograph was connected with an Autolab integrator type 6300.

6.3 Results

A representative example of the time course of the absorbance change at 518 nm (ΔA518) upon a single turnover light flash in dark-adapted broken chloroplasts, isolated from plants grown at a light intensity of 60 $W.m^{-2}$, is illustrated in Fig. 6.1 (solid curve).

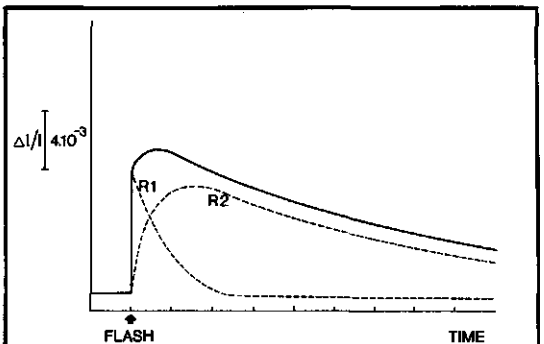

Fig. 6.1. Absorbance changes at 518 nm in dark-adapted broken chloroplasts induced by a single-turnover light flash (solid-curve). Chloroplasts were isolated from spinach plants grown at a light intensity of 60 $W.m^{-2}$. The measurement was performed in a medium containing Hepes-KOH 50 mM (pH 7.5), sorbitol 330 mM, $MgCl_2$, 2mM, $MnCl_2$ 1 mM, and EDTA 2 mM, at chl = 30 μg/ml. Average of 8 flashes fired at a rate of 0.10 Hz.

From this figure it can be seen that ΔA518 under these conditions occurs with multiphasic rise and decay kinetics. By using double flashes (chapter 3 of this thesis) it has been shown that the single-flash response curve can be deconvoluted into three separate responses: (a) a fast response (reaction 1) with a fast rise (0.5 ms) and a single exponential dark decay with a half-life of about 75 ms; (b) a slow response (reaction 2) with a slow rise (within 150 ms after the flash) and a slow single exponential decay with a half-life of about 500 ms; and (c) a relatively small phase-d with an extremely slow decay (half-life of about 1500 ms). These responses (i.e., reactions 1 and 2), determined in our experiments according to the aforementioned procedure, are indicated in Fig. 6.1 by the dashed curves (phase-d not shown). From this figure, it can be seen that the decay of the overall P515 response is almost exclusively determined by the decay of reaction 2. Figure 6.2 shows the absorbance change at 518 nm upon a single turnover light flash in dark-adapted broken chloroplasts isolated from plants grown at a light intensity of 6 $W.m^{-2}$ (solid curve). From this figure it can be seen that the contribution of reaction 2 to the overall kinetics of the P515 response in these chloroplasts is largely reduced. Whereas the rise and decay kinetics of reaction 2 itself are not altered significantly, the contribution of reaction 2 to the overall P515 response is reduced to about 25% in low-light chloroplasts as compared to the ap-

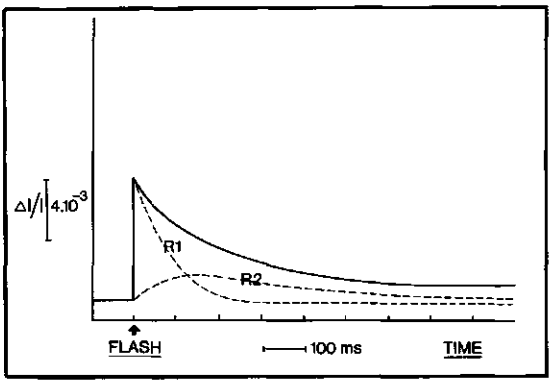

Fig. 6.2. Absorbance changes at 518 nm in dark-adapted broken chloroplasts induced by a single-turnover light flash (solid-curve). Chloroplasts were isolated from spinach plants grown at a light intensity of 6 $W.m^{-2}$. Average of 9 flashes fired at a rate of 0.10 Hz. Conditions as in Fig. 1.

parent high contribution in chloroplasts isolated from high-light grown plants. No significant differences between these two types of chloroplasts could be detected with respect to the kinetics of reaction 1.

Table I gives the results of a comparative analysis of these two types of chloroplasts with respect to some of the components involved in the functional and structural organization of the photosynthetic electron transport system.

Table I. Molar Ratios of Chlorophyll a/b Chlorophyll/P_{700}, and Chlorophyll/cyt b_{563} + cyt f (Relative Units), and the Values for the Hill Reaction Rate and the Size of the PQ Pool Relative to the Primary Acceptor Q_A, Determined in Chloroplasts Isolated from Plants Grown at a Light Intensity of 60 and 6 $W.m^{-2}$, Respectively.

	Chlorophyll a/b	Hill reaction µmol O_2. mg chl^{-1} .hr^{-1}	Chl / P_{700}	PQ /Q_A	Chl/cyt b_{563} +cyt f arbitrary units
Chloroplasts 60 Wm^{-2}	2.8±0.1	190±10	490±10	12±1	100
Chloroplasts 6 Wm^{-2}	2.5±0.1	155±10	510±10	12±1	250

From the table it can be seen that there are no significant differences between these two types of chloroplasts with respect to the ratios of the molar concentrations of chlorophyll a and b, chlorophyll and P700, as well as for the size of the PQ pool. Figure 6.3 shows the fluorescence induction curves for the two different types of chloroplasts in the presence and absence of 2 µM DCMU.

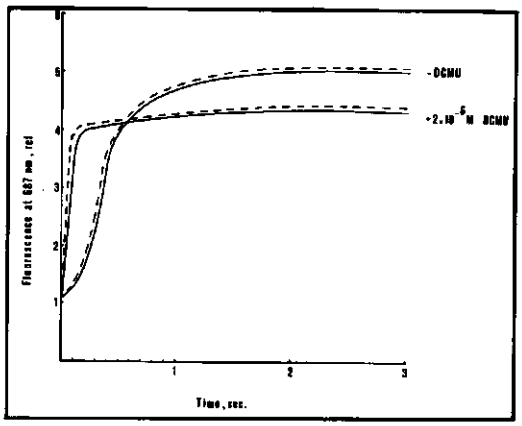

Fig. 6.3. Fluorescence kinetics at 20° C of chloroplasts isolated from spinach plants grown at 60 $W.m^{-2}$ (dashed curve) and at 6 $W.m^{-2}$ (solid curve). The induction curves were determined after a period of 90 min dark adaptation in the presence and absence of DCMU, in a medium containing sorbitol 330 mmol/liter, Hepes-KOH 50 mmol/liter, pH 7.4, $MgCl_2$ 1 mmol/liter, and 20 µg chl.ml. Fluorescence (λ687 nm) was excited with high-intensity light (λ380-550 nm).

Both types of chloroplasts show the same induction kinetics, and the amount of electrons that can be stored in the PQ pool, calculated by comparing the areas above the induction curves in the presence and absence of DCMU, appears to be about 12 for both samples. In contrast with this, the summed amount of cyt f and cyt b_{563} in chloroplasts isolated from plants grown at 6 $W.m^{-2}$ appeared to be reduced to about 40% of the values measured in chloroplasts isolated from plants grown at 60 $W.m^{-2}$ (see Fig. 6.4).

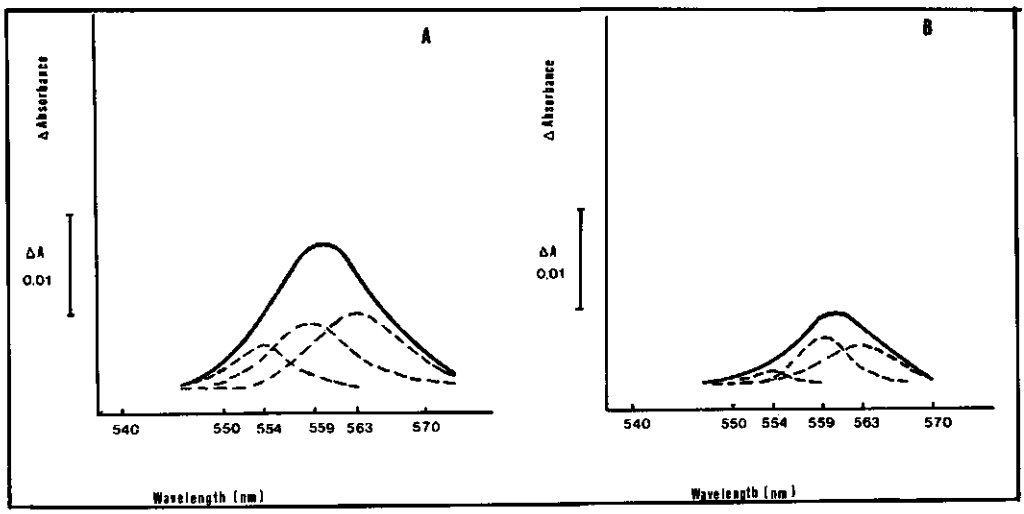

Fig. 6.4. Dithionite-reduced minus ferricyanide-oxidized difference spectrum of cytochromes in chloroplasts isolated from plants grown at 60 $W.m^{-2}$ (A) and 6 $W.m^{-2}$ (B), respectively. The dotted lines represent the calculated contribution of the different types of cytochromes to the overall spectrum.

From these experiments, the quantitative differences for each type of cytochrome could not be determined with sufficient precision. However, our data appear to be conclusive with a relative concentration ratio P700:cyt f:cyt b_{563} of 1:1:2. The reduction of the total amount of both cyt f and cyt b_{563} in low-light chloroplasts is coincident with a 20% reduced rate of the Hill reaction. Table II reports on the lipid composition of stripped (i.e., envelope-free) thylakoids isolated from plants grown at 6 and 60 $W.m^{-2}$, respectively.

Table II. Lipid Class Composition of Thylakoid Membranes Isolated from Plants Grown at Different Light Intensities.

Sample:	mol lipid / mol tot.chl.	Lipid class composition (mol %)				MGDG / DGDG
		MGDG	DGDG	PL		
60 W/m^2	2.42	53.2	36.5	10.3		1.46
6 W/m^2	1.46	47.5	46.5	6.0		1.02

From the table, an appreciable difference in the lipid composition between these two types of chloroplasts can be observed. In chloroplasts isolated from plants grown at a light intensity of 6 $W.m^{-2}$, the ratio of the two major membrane lipids, i.e., monogalactosyldiacylglycerol to digalactosyldiacylglycerol, is reduced to about 65% of the value found in high-light chloroplasts.

6.4 Discussion

On the basis of the kinetics of reaction 2 we suppose, in conformation with others (18-20) that this reaction is not directly linked to a transmembranal potential but rather to an intramembranal local field. In particular, the slow decay associated with reaction 2 is an indication that this reaction is associated with an electral event that takes place in an area, presumably a hydrophylic phase adjacent to or inside the membrane, which is not in direct equilibrium with the inner or the outer aqueous phase. In contrast to reaction 1, the occurrence of reaction 2 is strongly

dependent on the state of activation of the thylakoid membrane. Reaction 2, most obvious in dark-adapted chloroplasts, is largely reduced or even absent from the P515 response under conditions at which the thylakoid membrane is energized preceding the actinic light flash. This suppression of reaction 2 can be brought about either by illumination (i.e., the second slow rise is largely absent in the P515 response of a single flash following two preceding light flashes separated in time for 100 ms) or by an activated ATPase (see chapter 3).

Obviously, the proposed inner-membranal electric fields associated with reaction 2 can be created by light-driven electron transport as well as by reversed electron flow caused by ATP hydrolysis in the dark. The energetic requirement to generate these fields apparently is low, because of the small number of single turnover light flashes required to saturate reaction 2, and the long periods at which the saturation can be sustained by an activated ATPase (chapter 3).

It has been proposed that reaction 2 is caused by lateral and transverse delocalization of inner-membrane electric fields associated with the liberation of protons in inner-membrane domains near the Fe-S cyt b-f protein complex (1). In this respect it is of interest that the suppression of reaction 2 in chloroplasts isolated from plants grown at a light intensity of 6 $W.m^{-2}$ (Fig. 6.2) is associated with a 60% reduction of the amount of cyt f and cyt b_{563} (Table I). This reduction was found to have much less consequence for the rate of photosynthetic electron transport, as is indicated by the approximately 20% reduction of the Hill reaction in these chloroplasts (Table I). This indicates a low flux control of the electron transport rate by the cyt b-f complex, as was suggested by Kackser (21). The existence of these proposed intramembrane structures capable of stabilizing protons within the hydrophobic matrix of the membrane remains to be demonstrated.

As can be seen from Table II, the apparent reduction of reaction 2 in the P515 response in chloroplasts isolated from plants grown at a light intensity of 6 $W.m^{-2}$ is associated with an appreciable reduction of the ratio of the two major membrane lipids, monogalactosyldiacylglycerol to digalactosyldiacylglycerol. This reduced ratio was due mainly to a reduced amount of MGDG in the low-light chloroplasts. The difference in the ratio of MGDG to DGDG between chloroplasts isolated from plants grown at low and high light intensities, respectively, is probably associated with a dif-

ference in the stage of development between these two groups of plants at the moment of harvesting (7 weeks after sowing). Whereas the plants grown at a light intensity of 60 $W.m^{-2}$ had, at the moment of harvesting, six fully expanded green leaves, the plants grown at a light intensity of 6 $W.m^{-2}$ had only two. It has been reported that the MGDG/DGDG ratio is dependent on the stage of development of the plants, i.e., is low in the initial stages (22). The apparent low amount of MGDG in thylakoid membranes isolated from plants grown at a low light intensity may affect the architecture and the structural organization of the membrane. As reported by Shipley (23) isolated MGDG dispersed in aqueous environments tends to form preferentially nonbilayer structures, described as a hexagonal type-II phase. Such nonbilayer structures adopted by the MGDG molecules could theoretically create hydrophilic microcompartments in the hydrophobic membrane that are able to stabilize protons or, in combination with proteins, protonated residues. This tendency of MGDG to form nonbilayer structures, at least in vitro (24,25), may have an impact on the heterogeneous organization of the thylakoid bilayer in vivo. According to Israelachvili (26) and Gounaris (27) cone-shaped molecules like MGDG may serve conditions of a special packaging of large protein complexes in biological membranes. Thus, one might suggest that a reduced amount of MGDG in thylakoid membranes will alter the assembly of protein complexes in the membrane. This will, among others, affect the proton permeability of the membrane. If protons were stabilized within hydrophilic microcompartments adjacent to or inside the membrane, the stabilizing ability is reduced when the proton permeability is enhanced. This means that after proton loading, the microcompartments would equilibrate with the lumen at a higher rate and as a consequence, the extent of reaction 2 is smaller.

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CHAPTER 7, THE EFFECT OF UNCOUPLERS (F) CCCP AND NH_4Cl ON THE KINETICS OF THE FLASH-INDUCED P515 ELECTROCHROMIC BANDSHIFT IN SPINACH CHLOROPLASTS.

In collaboration with O. van Kooten and W.J. Vredenberg. Modified version of publication in FEBS Letters, Vol. 177, No. 1 (1984)11-16.

Abstract

The effect of uncouplers of photophosphorylation on the kinetics of the flash-induced P515 electrochromic bandshift was investigated in dark-adapted chloroplasts. It was found that the presence of low concentrations of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) resulted in the selective suppression of the slow P515 absorbance change (reaction 2), whereas the fast change (reaction 1) was not influenced. In contrast, high concentrations of NH_4Cl did not alter the P515 response with respect to reactions 1 and 2. These results indicate that reaction 2 is specifically sensitive towards uncouplers which exert their function as a proton carrier in the lipophilic phase of the membrane.

7.1 Introduction

As was stated before, the reaction 2 component of the flash-induced P515 response is thought to be related to intramembranal electrical phenomena (chapter 5 and 6, refs. 1-3), presumably associated with the liberation of protons in inner-membrane domains near the cytb-f protein complex. Although the exact nature of these intramembranal electric fields is still unclear, their appearance, as reflected by reaction 2, has been found to be strongly dependent on the functional integrity of the membrane. Reaction 2 disappears from the P515 response in chloroplasts upon ageing, after a temperature shock, and as we have shown in chapter 6, is largely reduced in chloroplasts isolated from plants which were grown at low light intensity. It was found (chapter 6) that these plants showed an alteration with respect to the lipid composition of the thylakoid membrane as well as a largely reduced amount of $cytb_{563}$ and cytf. Here, we present results in support of our suggestion that the reaction 2 is associated with the liberation and subsequent stabilization of protons inside the lipophilic phase of the thylakoid membrane. We have investigated the effects of two types of uncouplers of photophosphorylation with a different mode of action, on the kinetics of the flash-induced P515 response. It was found that in the presence of low concentrations of FCCP and CCCP (10^{-7} and 10^{-6} M, respectively), both of which are known to act as proton carriers in the lipophilic phase of the membrane, the reaction 2 component in the P515 response is

selectively reduced. At these low concentrations, hardly any effect was found on the Hill reaction rate and the kinetics of reaction 1. In contrast, the addition of 5 x 10^{-3} M of the uncoupler NH_4Cl did not result in an alteration of the P515 response, with respect to reactions 1 and 2. At this concentration the Hill reaction was shown to be highly uncoupled. Obviously, the uncoupler NH_4Cl, that acts as a proton binding agent in the hydrophilic phase (i.e., the lumen) of the thylakoid, has no effect on the reaction 2 component of the P515 response. From these experiments it is concluded that the reaction 2 component of the P515 response is selectively sensitive towards uncouplers of photophosphorylation that act as a proton carrier in the lipophilic phase of the membrane. These results are in support of our suggestion that reaction 2 is the reflection of an intramembranal electrical event that is associated with the liberation and subsequent stabilization of protons in inner-membrane domains. This stabilizing ability is lost upon the addition of a lipophilic protonophore.

7.2 Materials and Methods

Freshly grown spinach (Spinacia oleracea cv: Bergola) was used for all experiments. The plants were grown under high-pressure mercury lamps (Philips MGR 102-400) at an intensity of approx. 100 $W.m^{-2}$ with a light period of 8 h per day. Provisions were made to keep the temperature at the leaf and soil surface at 18-20° C. The relative humidity of the atmosphere was minimal 70%. Intact chloroplasts were routinely isolated according to a modified method of (5) as described in chapter 2. This procedure routinely yielded preparations with 90-95% intact chloroplasts as determined by ferricyanide reduction (6). Absorbance changes at 518 nm induced by single turnover flashes in intact chloroplasts were measured in a modified Aminco chance absorption difference spectrophotometer as described in chapter 2. The reaction medium contained: 50 mM Hepes-KOH (pH 7.5), 330 mM sorbitol, 2 mM $MgCl_2$ and 1 mM $MnCl_2$. FCCP (Sigma C 4017) and CCCP (Sigma C 2759) were added to the sample from stock solutions containing 80% ethanol. The ethanol concentration in the reaction medium never exceeded 2%. The Hill reaction in intact chloroplasts was determined as the consumption of oxygen in the presence of 2 x 10^{-5} M methyl viologen as an electron acceptor. The oxygen consumption was measured with a Gilson oxygraph as in (7).

7.3 Results and Discussion

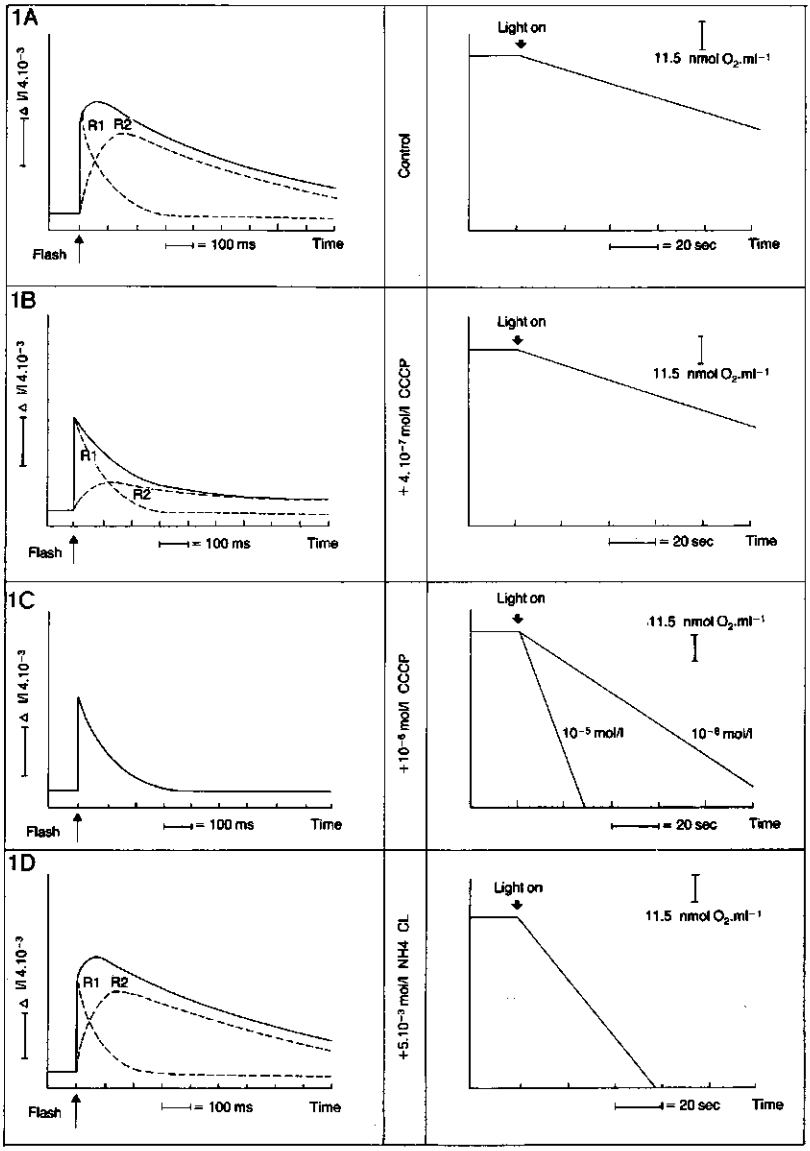

Fig. 7.1. Left-hand side: Absorbance changes at 518 nm in dark-adapted intact chloroplasts induced by a single-turnover light flash (———) and the deconvolution of the overall signal into reactions 1 and 2 (- - - →) in the absence (a) and presence of 4 X 10^{-7} M and 10^{-6} M CCCP (b c, respectively) and 5 X 10^{-3} M NH_4Cl (d). Right-hand side: Rate of oxygen consumption in continuous illumination in the absence (a) and presence of CCCP (b,c) and NH_4Cl (d).

A representative example of the time course of the absorbance change at 518 nm (ΔA_{518}) upon a single turnover light flash in dark-adapted and well preserved chloroplasts is illustrated in fig. 7.1A. From this it can be seen that ΔA_{518} under these conditions occurs with multi-phasic rise and decay kinetics. By using double flashes it has been shown (ref. 1, chapter 3) that the single flash response curve can be deconvoluted into at least two separate responses, reactions 1 and 2. These responses (i.e., reactions 1 and 2), determined in our experiments according to the aforementioned procedure, are indicated in the figure by the dashed curves. The effect of the addition of 4×10^{-7} M CCCP to a sample of intact chloroplasts on the kinetics of the P515 response and on the Hill reaction rate is shown in fig. 7.1B. From this it can be seen that the contribution of the reaction 2 component to the P515 response is reduced to about 30% compared to the value found in the absence of CCCP, whereas the kinetics of reaction 1 and the Hill reaction rate are hardly affected.

Raising the concentration of CCCP to 10^{-6} M (fig. 7.1C) results in the complete suppression of reaction 2 from the P515 response. At this concentration, the kinetics of the P515 response are exclusively determined by the reaction 1 component and can be characterized by a fast rise in absorbance and a subsequent single exponential dark decay with a rate constant of about 10 s^{-1}. As shown in fig. 7.2, progressive addition of CCCP to a sample of intact chloroplasts in the range 10^{-8}–10^{-6} M results in the selective suppression of reaction 2 from the P515 response, whereas in this range only minor effects can be detected on the kinetics of reaction 1 and on the Hill reaction rate.

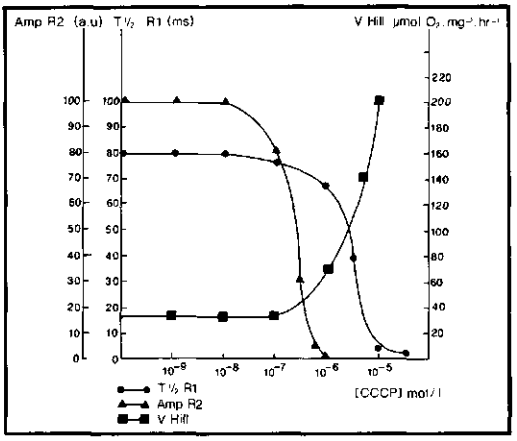

Fig. 7.2. The amplitude of reaction 2 (▲—▲) the half-lifetime of reaction 1 (●—●) and the Hill reaction rate (■—■) in intact chloroplasts as a function of CCCP concentration.

These rates become altered at concentrations above 10^{-6} M. The effect of CCCP was found to be reversible. After CCCP was removed from the membrane by the addition of BSA, reaction 2 appeared to be completely restored (not shown). Qualitatively, the same results were found for the uncoupler FCCP. Also in the presence of this protonophore, reaction 2 was shown to be completely suppressed at low concentrations (10^{-7} M) whereas the reaction 1 component and the Hill reaction were only slightly affected (not shown). As can be seen from fig. 7.1D and fig. 7.3, the addition of 5 x 10^{-3} M NH_4Cl to a sample of intact chloroplasts did not alter the kinetics of the P515 response, with respect to reactions 1 and 2.

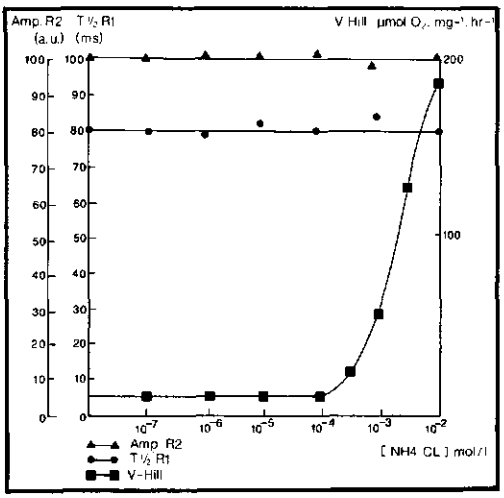

Fig. 7.3. The amplitude of reaction 2 (▲—▲), the half-lifetime of reaction 1 (●——●) and the Hill reaction rate (■—■) in intact chloroplasts as a function of HN_4Cl concentration.

However, the Hill reaction was highly uncoupled at this concentration of NH_4Cl. Obviously, reaction 2 is selectively sensitive towards uncouplers of photophosphorylation that act as proton carriers in the lipophilic phase of the thylakoid membrane (e.g., CCCP and FCCP). Moreover, reaction 2 is not influenced by the uncoupler NH_4Cl that has a function in the dissipation of the transmembrane proton gradient by binding protons in the hydrophilic (i.e., the luminal) phase of the thylakoid interior.

It was suggested earlier (chapter 5 and 6) that reaction 2 is the reflection of an intramembranal electrical event that is associated with the liberation and subsequent stabilization of protons in inner-membrane domains, presumably near the Fe-S cytb-f protein complex. These domains have been suggested to be connected, via lateral H-conductive channels,

with other membrane domains that act as proton sinks (i.e., the ATP synthease). Models depicting site specific intramembranal proton processing in the thylakoid have been suggested (8.9).

Although direct evidence for the stabilization of protons in domains inside or near the hydrophobic membrane is still lacking, it must be stated that the occurrence of reaction 2 is strongly dependent on the functional integrity of the membrane, i.e., disappears upon ageing, after a temperature shock and as was shown in chapter 6, is largely reduced in chloroplasts isolated from plants which were grown at low light intensity. It was found that these plants showed an alteration in the lipid composition of the thylakoid membrane as well as a largely reduced amount of cyt b_{563} and cytf (chapter 6).

If protons were stabilized within small hydrophilic domains adjacent to or inside the membrane, the stabilizing ability thereof is expected to be reduced when the proton permeability is enhanced. This will happen in the presence of lipophilic protonophores like CCCP and FCCP, which act as proton carriers in the lipophilic phase of the thylakoid membrane. As a consequence, the equilibration of the proposed proton pools with the luminal phase will be enhanced in this case and, as indeed observed, the extent of reaction 2 will be smaller. At the concentrations needed to suppress completely the reaction 2 component of the P515 response (10^{-6} and 10^{-7} M for CCCP and FCCP, respectively) hardly any effect could be measured on the rate of the Hill reaction. Obviously, at these low concentrations the protonophoric effect of the agents is still too low to affect a still coupled electron transfer rate, presumably because the transmembrane proton gradient, sustained by continuous illumination, is hardly altered, if at all. The unaltered decay rate of reaction 1 under these conditions is consistent with this. On the contrary, the addition of 5 mM NH_4Cl did not result in an alteration of the kinetics of the P515 response, with respect to reactions 1 and 2, whereas the Hill reaction was shown to be effectively uncoupled at this concentration. Apparently the hydrophilic nature of this uncoupler does not meet the conditions for destabilization of protons in membrane domains, i.e., inhibition of reaction 2. Therefore, these domains are likely to be of a lipophilic character.

These results are conclusive with an energy conserving mechanism in which localized protons exert a subtle membrane control pattern in the activity of the ATP synthetase. The slow component of P515 (reaction 2)

appears to be a promising tool to study further details and physiological aspects of this control mechanism in intact membrane systems under various conditions.

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CHAPTER 8, GENERAL DISCUSSION.

General discussion

The experimental results presented in this thesis are a contribution to the discussion about the origin of the slow component in the P515 response. In this chapter these results will be discussed in relation to the dynamic function of the thylakoid membrane in energy transduction and energy coupling. Alternative interpretations given by other authors will be discussed in connection with our results.

A major problem when studies of the flash-induced P515 response in intact leaves or isolated chloroplasts are made, is the fact that the slow response (i.e. reaction 2) is not detectable under many experimental conditions. Reaction 2, normally present in dark-adapted membranes, disappears from the signal after short periods of preillumination, after ageing and after a temperature shock. Moreover, the isolation procedures followed are determinant for the quality of the isolated chloroplasts and, as a consequence, determinant for the occurrence of reaction 2. Because of this it is not surprising that discrepancies can occur between the experimental results reported by different investigators. In our experiments, efforts have been made to optimalise growth conditions of the plant material and isolation procedures of chloroplasts, in order to obtain leaves and chloroplasts which possess a P515 response with a constant and relative high contribution of reaction 2. As a consequence, in this material reaction 2 can be measured without the need for artificial reducing agents for the photosynthetic system as is sometimes reported in the work of others (1).

On the basis of the kinetics of reaction 2 we suppose, in conformation with others (2-4) that this reaction is not directly linked to a transmembranal electric potential but rather to an intramembranal local electric field. After analysis (see chapter 3), reaction 2 can be characterized by a relatively slow increase in absorbance during the first 20 to 100 msec after the flash, followed by a decay with a half life of about 400 msec. In particular the slow decay associated with reaction 2 is an indication that this reaction takes place in an area, presumably a hydrophilic phase adjacent to or inside the membrane, which is not in direct equilibrium with the inner or the outer aqueous phase. Such a local domain is expected to have a limited electrical capacity. This is confirmed by the apparent low energetic requirement to generate these fields, i.e. the small number of single saturating light flashes required to saturate reaction 2, and the

long periods at which it can be sustained by an activated ATPase (chapter 3). From the results in chapter 3 it can be concluded that the electric fields associated with reaction 2 can be created either by light-driven electron transport or by reverse electron flow caused by ATP hydrolysis.

It has been suggested by Van Kooten (5) that reaction 2 is caused by lateral and transverse delocalization of inner-membrane electric fields associated with the liberation and subsequent stabilization of protons in inner-membrane domains near the Fe-S cyt b-f protein complex. The consequence of hindered diffusion as suggested by others (6) on the formation of isopotentials under continuous illumination have been simulated in a model (7). There appears a reasonable agreement with the experimental data and the simulation model. We suggest that these domains might be connected via lateral H-conductive channels with other membrane domains which act as proton sinks (i.e. ATP synthetase). This is in accordance with the fact that the slow light- and ATP synthetase-induced electric potential change are of the same nature and in fact fully complementary as is shown (in conformation with results of others (3,4)) in chapter 3. Models depicting site-specific intramembranal proton processing in the thylakoid have been suggested by Dilley (8) and by Kell (9). Considering this, the occurrence of reaction 2 in the P515 response may be correlated with the state of activation of the thylakoid membrane and, in this way, might be of relevance to a localized chemiosmotic coupling mechanism. Under conditions at which the membrane is energized and, as a consequence, reaction 2 cannot be seen (as for instance under conditions at which the ATPase is activated and ATP hydrolysis takes place), flash-induced ATP synthesis can be expected to occur (see chapter 4). However, endogeneous factors determining ΔG_{ATP} in the dark (such as adenylate kinase activity) should be taken into account when studies of the kinetics of initial ATP synthesis are performed.

Ample evidence has been presented, both in chloroplasts and in green algae, that the overall decay rate of the flash-induced P515 response is substantially accelerated after short periods of illumination (10-12). It has been concluded, following the interpretation of others (13,14), that the decay rate is mainly determined by the H^+-efflux through the ATPase, i.e. is higher under conditions at which the chloroplast ATPase is activated. In accordance with this, Morita et al. (15) demonstrated a seeming connection between the activity of the ATPase and the apparent P515 decay rate. From the experiments in chapter 5 however, it is concluded that

reduction of the H^+-permeability of the thylakoid membrane-bound ATPase caused by DCCD has no effect on the kinetics of the P515 response induced by a light flash. The observed acceleration of the overall decay rate of the P515 response can be explained by the suppression of the reaction 2 component of the response which takes place under conditions where the thylakoid membrane is energized. The duration of the suppression period however, is found to be significantly reduced in the presence of DCCD. This finding is important for the interpretation of the experimental results reported by Morita. In Morita's experiments, the flash-induced absorbance change was measured 90 to 170 sec after the illumination period. In chapter 5 it is shown for chloroplasts, that the suppression of reaction 2 in the presence of DCCD is sustained for a maximum period of 90 sec. This means that after 90 sec dark adaptation the P515 response induced by a light flash is found to be identical (i.e. includes reaction 2) to the response obtained before the illumination period. Obviously, the results reported by Morita can be explained by the relative long period of dark adaptation preceding the actinic light flash. From these experiments we conclude, in conformation with others (2) that protons do not contribute significantly to the membrane conductance, even under phosphorylating conditions.

As was found by others (16), the occurrence of reaction 2 in the flash-induced P515 response is strictly dependent on the functional integrity of the membrane, i.e. it disappears upon ageing and is absent in chloroplasts that have been rethawed from a frozen preparation. As is shown in chapter 6, reaction 2 is also largely reduced in plants grown at low light intensities. This significant reduction of reaction 2 in the P515 response has been found to be coincident with a 35% reduced ratio of the two major membrane lipids, MGDG to DGDG. The reduced ratio is mainly due to a reduced amount of MGDG in the low-light chloroplasts. The tendency of MGDG to form non-bilayer structures, at least in vitro (17,18), may have an impact on the heterogeneous organization of the thylakoid in vivo. According to Israelachvili (19) and Gounaris (20), cone-shaped molecules like MGDG may serve conditions of a special packaging of large protein complexes in biological membranes. Thus, one might suggest that a reduced amount of MGDG in thylakoid membranes will alter the assembly of protein complexes in the membrane. This will, amongst others, affect the proton permeability of the membrane. If protons were stabilized within hydrophilic microcompartments adjacent to or inside the membrane, the stabilizing ability is re-

duced when the proton permeability is enhanced. This means that after proton loading, the microcompartment will equilibrate with the lumen at a higher rate and as a consequence, the extent of reaction 2 is smaller. It is also found (chapter 6) that the reduction of reaction 2 in the P515 response is coincident with a largely reduced amount of both cytochrome b_{563} and cytochrome f. This result is the more interesting since it has been suggested by others (21-24) that reaction 2 results from redox reactions in the Qbc region of the electron transport chain. An association with cyt.b_{563} reoxidation has been suggested by Selak and Whitmarsh (25). However, it has been shown (26) that the seeming correspondence between the potential associated with this secondary electron transport and a turnover of cyt b_{563} only holds for a single flash. A second turnover of cyt-b6 induced by a second flash, given 100 msec after a first one does not, or at least much less, cause a slow rise in the flash-induced P515 response. Moreover, the occurrence of a slow phase in the rise of the P515 response is obligatory associated with a slow phase in decay kinetics. The interpretation of Selak does not account for the fact that the field generated by this secondary electron transfer decays with a half time which is considerably higher than the decay of the potential generated by electron transfer through the reaction centers (2). In recent experiments on the origin of the electrogenic reaction in the chloroplast cytochrome b/f complex (27) the loss of the slow rise in the P515 response is not accompanied with an acceleration of the overall decay rate. However, in these experiments, the overall decay of the P515 response has a half life of about 50 msec and it is assumed by the authors that the slow phase in the P515 response decays with the same half time as the fast response. As stated before, we find, in conformation with others (4, 28), that the occurrence of the slow rise in the P515 response is obligatory associated with a slow phase (half life of about 400 msec) in the decay kinetics.

Additional support for our suggestion that the reaction 2 component of the flash-induced P515 response is associated with the liberation and subsequent stabilization of protons inside a lipophilic phase of the thylakoid membrane is given in chapter 7. It is found that in the presence of low concentrations of FCCP and CCCP, both of which are known to act as a proton carrier in the lipophilic phase of the membrane, the reaction 2 component in the P515 response is selectively reduced. In contrast, the addition of concentrations of NH_4Cl sufficiently high to uncouple the Hill

reaction, does not result in an alteration of the P515 response. Obviously, the uncoupler NH_4Cl that acts as a proton binding agent in the hydrophilic phase (i.e. the lumen) of the thylakoid, has no effect on the reaction 2 component.

From the results presented in this thesis there is accumulating evidence that reaction 2 has its origin in innermembrane processes which are dependent on the existence of domains which facilitate proton interaction in the lateral plane of the membrane and may give rise to localized chemiosmotic coupling mechanisms, and electrochromic effects on the native P515 pigment complex.

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Summary

The main part of this thesis deals with studies and analyses of the kinetics of the flash-induced P515 electrochromic bandshift in spinach leaves and isolated chloroplasts thereof. The kinetics of the P515 response are discussed in relation to the dynamic function of the thylakoid membrane in energy transduction and energy coupling. The ultimate aim of the studies reported in this thesis is to obtain more information about the nature and physiological origin of the slow component (i.e. reaction 2) of the flash-induced P515 response.

A general introduction to the mechanism of photosynthetic energy conversion is given in chapter 1. Also in this chapter some characteristics of the P515 pigment complex as an intrinsic, field sensitive probe are discussed.

Methods for the determination of the flash-induced absorbance difference of P515, the adenylate kinase activity, ATP hydrolysis, membrane lipid composition and compounds involved in the functional and structural organization of the photosynthetic electron transport system are described in chapter 2.

In chapter 3 the results of a comparative study of the effects of preillumination on the kinetics of the P515 response and on the activity of the chloroplast ATPase are presented. It is shown that energization of the membrane, either by light-driven electron transport or by reverse electron transport induced by ATP hydrolysis, results in the specific suppression of reaction 2 in the flash-induced P515 response. Qualitatively the same results are found in subchloroplast vesicles following energization of the membrane by either preillumination or by an artificially induced transmembrane pH gradient. In chloroplasts, under conditions in which the ATPase is activated and ATP hydrolysis can be measured, reaction 2 of the P515 response induced by a single light flash is largely if not completely suppressed. This suppression is demonstrated to be reversible, and the duration of the suppression period appeared to be coincident with the period of time ATP hydrolysis can be detected. The results in this chapter do show that reaction 2 can also be induced in the dark toward its saturation level by ATP-driven reverse electron flow.

The activity and behaviour of the chloroplast ATPase in spinach chloroplasts after single turnover light flashes has been studied in chapter 4.

It has been found that endogeneous factors determining $\Delta GATP$, such as of the adenylate kinase, must be considered when the kinetics of initial ATP synthesis and of P515 in light flashes are studied.
In dark-adapted chloroplasts with an endogeneous high adenylate kinase activity, the first 4 flashes cause ATP hydrolysis which is shown to persist for an extended dark period. During this period the membrane remains energized and, consequently, reaction 2 in the flash-induced P515 response is fully suppressed. In chloroplasts showing low adenylate kinase activity, ATP hydrolysis can only be induced by a successive flash train of at least 60 flashes or by a 30 to 60 sec period of continuous preillumination. These results explain the fact that in spinach varieties showing high adenylate kinase activity (for instance in summer spinach varieties grown under suboptimal light conditions) the flash-induced P515 electrochromic bandshift determined in intact leaves has been found to be devoid of reaction 2.

Chapter 5 deals with the effect of the energy transfer inhibitor compound DCCD on the kinetics of the flash-induced P515 response and on the activity of the ATPase. It is shown that concentrations of DCCD sufficiently high to affect the ATPase activity, have no effect on the kinetics of the P515 response. Obviously, the reduction of the H^+-permeability of the thylakoid membrane-bound ATPase caused by DCCD has no effect on the kinetics of the P515 response. Therefore, we conclude that this permeability for protons is not a determinant factor in the regulation of the flash-induced membrane potential and does not affect the decay rate of the P515 response induced by a single turnover light flash. The apparent acceleration of the overall decay rate of the flash-induced P515 response found after short periods of illumination can be fully explained by the fact that the reaction 2 component is suppressed as a consequence of energization of the membrane by an activated hydrolizing ATPase.

The results presented in chapter 6 indicate a possible correlation between factors determining the structural organization of the thylakoid membrane and the kinetics of the flash-induced P515 response.

The significant reduction of reaction 2 in the P515 response observed in plants grown at low light intensities is shown to be coincident with a 35% reduced ratio of the two major membrane lipids, MGDG to DGDG. The reduced ratio is mainly due to a reduced amount of MGDG in the low-light chloroplasts.

Chapter 7 reports on an investigation of the effects of two types of

uncouplers of photophosphorylation with a different mode of action on the kinetics of the flash-induced P515 response. It is shown that in the presence of low concentrations of FCCP and CCCP, both of which are known to act as a proton carrier in the lipophilic phase of the membrane, the reaction 2 component in the P515 response is selectively reduced. At these low concentrations, hardly any effect was found on the Hill reaction rate and on the kinetics of reaction 1. In contrast, the addition of concentrations of NH_4Cl sufficiently high to uncouple the Hill reaction, do not result in an alteration of the P515 response, neither with respect to reaction 1 nor with respect to reaction 2. Obviously, the uncoupler NH_4Cl that acts as a proton binding agent in the hydrophilic phase (i.e. the lumen) of the thylakoid, has no effect on the reaction 2 component of the P515 response. From these experiments it is concluded that the reaction 2 component of the P515 response is selectively sensitive towards uncouplers that act as a proton carrier in the lipophilic phase of the membrane. These results are in support of the hypothesis that reaction 2 is the reflection of an intramembranal electrical event that is associated with the liberation and subsequent stabilization of protons in inner-membrane domains. This stabilizing ability is lost upon the addition of a lipophilic protonophore.

The results presented in this thesis are interpreted as evidence that reaction 2 has its origin in innermembrane phenomena which are dependent on the existence of lipid- and protein specific proton stabilizing domains which facilitate proton interaction in the lateral plane of the membrane and may give rise to localized chemiosmotic coupling mechanisms, and electrochromic effects on the native P515 pigment complex. The data presented illustrate the usefulness of the flash-induced P515 response as a qualifying monitoring probe for the energy requirement and maintenance of the activity of the membrane ATPase.

Samenvatting

In dit proefschrift worden de resultaten beschreven van een studie naar de kinetiek van het flits geinduceerde P515 signaal in spinazie bladeren en daaruit geisoleerde chloroplasten. De kinetiek van het P515 signaal zal worden besproken in relatie tot de functie van het thylakoid membraan bij het proces van energie-transduktie en energie-koppeling in chloroplasten. Het uiteindelijk doel van deze studies was nadere informatie te verkrijgen omtrent de betekenis van de langzame komponent (reaktie 2) in het flits-geinduceerde P515 signaal.

Een algemene introduktie van het mechanisme van fotosynthetische energie-konversie wordt gegeven in hoofdstuk 1. In dit hoofdstuk worden tevens enige eigenschappen vermeld van het P515 pigment komplex als intrinsiek veldgevoelige probe.

De methoden voor het meten van het flits geinduceerde P515 signaal, de aktiviteit van het enzym adenylaat kinase, ATP hydrolyse, de lipide samenstelling van het thylakoid membraan als ook de spectrofotometrische bepaling van componenten die betrokken zijn bij de functionele en strukturele organisatie van het fotosynthetisch electronentransport worden besproken in hoofdstuk 2.

In hoofdstuk 3 worden de resultaten gegeven van een vergelijkende studie naar het effect van voorbelichting op de kinetiek van het P515 signaal en de membraan gebonden ATPase. Het energetiseren van het thylakoid membraan, ofwel door licht opgewekt electronentransport, ofwel door "omgekeerd" electronentransport ten gevolge van ATP hydrolyse blijkt te resulteren in een specifieke suppressie van reaktie 2 in het flits geinduceerde P515 signaal. Kwalitatief gelijke resultaten werden gevonden in fotosysteem 1-verrijkte vesicles. Ook bij deze vesicles blijkt dat energetisering door middel van voorbelichting of door middel van een kunstmatig aangelegde membraan concentratiegradient van protonen, tot gevolg heeft dat specifiek reaktie 2 in het P515 signaal wordt onderdrukt. Deze onderdrukking van reaktie 2 blijkt van tijdelijke aard te zijn. In chloroplasten kan worden vastgesteld dat de lengte van de periode waarin reaktie 2 is onderdrukt samenvalt met de lengte van de periode waarin ATP hydrolyse kan worden gemeten. De resultaten in dit hoofdstuk geven aan dat reaktie 2 ook kan worden geinduceerd in het donker ten gevolge van ATP-gedreven "omgekeerd" electronentransport.

Het aktiveren van de chloroplast ATPase door "single turnover" licht flitsen wordt bestudeerd in hoofdstuk 4. In dit hoofdstuk wordt aangetoond dat endogene faktoren die van invloed zijn op $\Delta GATP$, zoals de aktiviteit van het enzym adenylaat kinase, in beschouwing genomen moeten worden wanneer studies naar de kinetiek van de initiatie van ATP synthese in lichtflitsen worden uitgevoerd. In donker geadapteerde chloroplasten die een hoge aktiviteit van het enzym adenylaat kinase vertonen blijken de eerste 4 flitsen een hydrolyse van ATP te veroorzaken. Deze periode waarin ATP hydrolyse kan worden aangetoond blijkt in het donker voor geruime tijd aan te houden. Gedurende deze periode blijft het membraan geenergetiseerd en kan derhalve reaktie 2 niet worden gemeten. In chloroplasten die een lage aktiviteit van het enzym vertonen blijkt ATP hydrolyse enkel te kunnen worden opgewekt door een flitstrein van tenminste 60 flitsen ofwel door een periode van continu belichting gedurende 30 a 60 seconden. Deze resultaten geven tevens een verklaring voor het feit dat in spinazie soorten die een hoge aktiviteit van het enzym adenylaat kinase vertonen (zoals bijvoorbeeld in zomersoorten die worden gekweekt onder sub-optimale lichtkondities) het flits-geinduceerde P515 signaal in bladeren meestal geen reaktie 2 bevat.

In hoofdstuk 5 wordt het effect onderzocht van de "energy transfer inhibitor" DCCD op de kinetiek van het P515 signaal en op de aktiviteit van de ATPase. Uit de resultaten van dit hoofdstuk blijkt dat concentraties DCCD die hoog genoeg zijn om de ATPase aktiviteit te remmen, geen effect hebben op de kinetiek van het P515 signaal. Kennelijk is de reduktie van de H^+-permeabiliteit van de membraan gebonden ATPase geen bepalende faktor voor de kinetiek van het P515 signaal. Wij konkluderen dan ook dat deze permeabiliteit voor protonen geen belangrijke faktor is in de regulering van de flits geinduceerde membraanpotentiaal, en geen invloed heeft op de afvalsnelheid van het P515 signaal. De versnelling van de afval van het overall P515 signaal, zoals dat wordt gevonden na korte periodes van belichting, kan volledig worden verklaard door het onderdrukken van reaktie 2 ten gevolge van een geaktiveerde ATPase.

De resultaten die worden gegeven in hoofdstuk 6 geven een mogelijk verband aan tussen faktoren die bepalend zijn voor de strukturele organisatie van het thylakoid membraan en de kinetiek van het flitsgeinduceerde P515 signaal. In dit hoofdstuk wordt aangetoond dat reaktie 2 sterk is gereduceerd in planten die zijn opgekweekt bij lage lichtintensiteit. Deze reduktie van reaktie 2 in het P515 signaal gaat samen met een 35% geredu-

ceerde ratio van de twee meest belangrijke membraan lipiden, MGDG en DGDG. Deze gereduceerde ratio wordt voornamelijk veroorzaakt door een verminderde hoeveelheid MGDG in chloroplasten uit planten die bij lage lichtintensiteit zijn opgekweekt.

In hoofdstuk 7 wordt een onderzoek naar de effecten van twee types ontkoppelaars op de kinetiek van het P515 signaal besproken. Het blijkt dat bij lage concentraties van CCCP en FCCP, welke beide werken als een protondrager in de lipofiele fase van het membraan, reaktie 2 selektief wordt onderdrukt. Bij deze lage concentraties kan nog nauwelijks enig effect op de Hill reaktie worden aangetoond. In tegenstelling hiermee blijkt dat het toevoegen van concentraties NH_4Cl die hoog genoeg zijn om de Hill reaktie te ontkoppelen, geen enkel effect hebben op de kinetiek van het P515 signaal. Kennelijk heeft de ontkoppelaar NH_4Cl, welke werkt als een proton bindend agens in de hydrofiele fase (het lumen) van het thylakoid, geen enkel effect op de kinetiek van het P515 signaal. Uit deze experimenten kan worden gekonkludeerd dat de reaktie 2 component van het P515 signaal selektief gevoelig is voor ontkoppelaars die werkzaam zijn als protondrager in de lipofiele fase van het membraan. Deze resultaten steunen onze suggestie dat reaktie 2 de reflektie is van een intramembraan electrisch veld dat is geassocieerd met het vrijkomen en vervolgens gestabiliseerd raken van protonen in speciale gebieden in het membraan. Dit stabiliserend vermogen gaat verloren als gevolg van het toedienen van een lipofiele protonofoor.

De resultaten in dit proefschrift vormen een toenemende mate van bewijs dat reaktie 2 zijn oorsprong vindt in een intramembraan electrisch veld. Dit veld is mogelijk geassocieerd met het bestaan van intramembraan gelegen domeinen die een rol spelen bij het vergemakkelijken van protoninterakties in het lateraal vlak van het membraan. Dergelijke laterale protoninterakties zijn mogelijk van belang voor een mechanisme van lokale chemiosmose en geven tevens aanleiding tot electrochrome effecten van het P515 pigment.

Curriculum vitae

Robert Peters werd geboren op 7 april 1956 te Arnhem. Na de lagere school werd de scholengemeenschap "Het Rhedens lyceum" in Rozendaal bezocht, waar in 1974 het Atheneum-B diploma werd behaald. In 1974 werd begonnen met een studie biologie aan de Rijksuniversiteit van Groningen. In 1978 werd het kandidaatsexamen B1 behaald en in 1981 het doctoraalexamen met als hoofdvakken microbiologie en plantenfysiologie. Van november 1981 tot januari 1985 ben ik via een beurs van SON verbonden geweest aan de vakgroep Plantenfysiologisch Onderzoek van de Landbouwhogeschool in Wageningen. Het onderzoek aan deze vakgroep, onder leiding van Prof. Dr. W.J. Vredenberg, heeft geleid tot het schrijven van dit proefschrift. Vanaf februari 1985 ben ik werkzaam bij ASTRA Pharmaceutica B.V. te Rijswijk.