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The Kinetics of the Flash-Induced P515 Response in Relation to the H^+-Permeability of the Membrane Bound ATPase in Spinach Chloroplasts

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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 5×10^{-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.

Key Words: P515; electrochromic band shift; ATPase; DCCD, proton flux; spinach chloroplast.

Introduction

The light-induced absorbance change around 518 nm is widely accepted as being the result of an electrochromic response of the P515[2] pigment complex to an electric field generated by the primary light-induced charge separation in the thylakoid membrane (Witt, 1979).

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[2] Abbreviations: P515: pigment complex with maximal absorbance change at 518; R1: reaction 1, R2: reaction 2; PSI: photosystem I; PSII: photosystem II; DCCD: dicyclohexylcarbodiimide; Fe–S: Rieske iron–sulfur protein; Cyt b–f: cytochrome b563–cytochrome f complex; CCCP: carbonylcyanide *m*-chlorophenylhydrazone; FCCP: carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DTE: dithioerythritol; ATP: adenosine-5′-triphosphate.

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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 (Joliot and Delosme, 1974; Morita *et al.*, 1981; Witt and Moraw, 1959). It has been concluded, following the interpretation of others (Girault and Galmiche, 1978; Junge *et al.*, 1970), 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.* (1982) 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 (1980), there is some doubt as to whether protons contribute significantly to the membrane conductance, even under phosphorylating conditions. According to Schapendonk *et al.* (1979), 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 vinicity 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 (Westerhoff *et al.*, 1983; Vredenberg, 1983).

It has been suggested (Dilley *et al.*, 1981) 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 (Schuurmanns *et al.*, 1981; Schreiber and Rienits, 1982), we have shown that reaction 2 can also be induced in the dark toward its saturation level by ATP-driven electron flow (proton translocation) (Peters *et al.*, 1983). 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 (Peters *et al.*, 1983). 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 (Peters *et al.*, 1983).

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.

Materials and Methods

Freshly grown spinach (*Spinacia oleracea*) 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 (Cockburn and Walker, 1968) as described by Schapendonk (1980). This procedure routinely yielded preparations with 90–95% intact chloroplasts as determined by ferricyanide reduction (Heber and Santarius, 1970). 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 518 nm induced by single turnover saturating light flashes in isolated chloroplasts were measured in a modified Aminco Chance absorption difference spectrophotometer as described before (Peters *et al.*, 1983). 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 before (Peters *et al.*, 1983).

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%.

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. 1A. From this figure it can be seen that ΔA518 under these conditions occurs with multiphasic rise and decay kinetics. By using double flashes it has been shown (Schapendonk *et al.*, 1979; Peters *et al.*, 1983) 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 (Westerhoff *et al.*, 1983; Vredenberg, 1983), 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 (Schapendonk, 1980; Vredenberg, 1983; Schuurmans *et al.*, 1981; Schreiber and Rienits, 1982) presumably associated with the liberation and subsequent stabilization of protons in innermembrane domains near the cyt b–f protein complex (Westerhoff *et al.*, 1983; Peters *et al.*, 1984a, b).

As can be seen from Fig. 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 before (Schapendonk, 1979; Peters *et al.*, 1983), 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. 1B and 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. 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 now shown). Figure 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

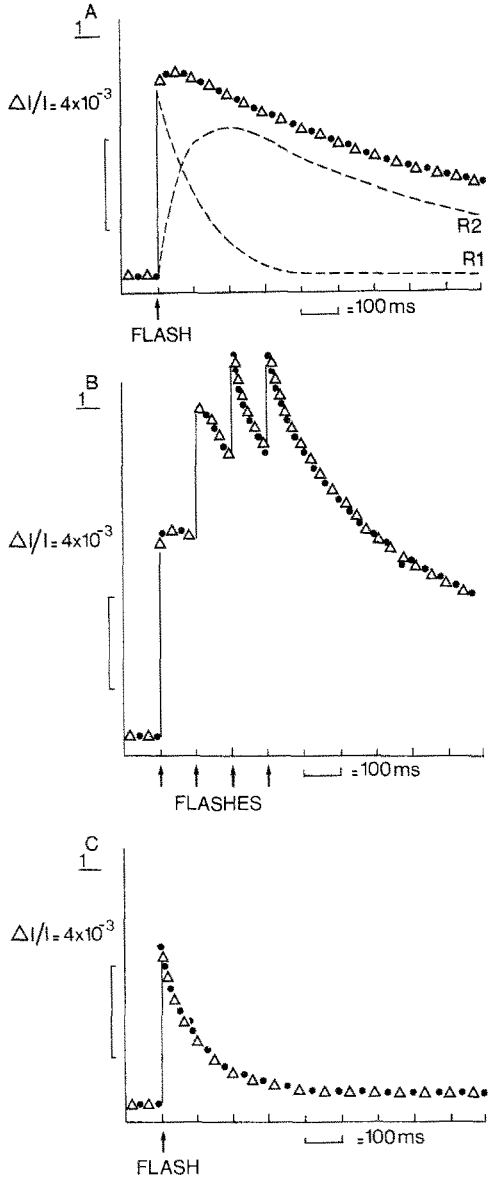

Fig. 1. Absorbance changes at 518 nm in dark-adapted broken chloroplasts induced by a single flash (A), and by a series of four successive flashes fired at 100-msec time intervals (B) measured in the absence (●) and presence (△) of 5×10^{-8} mol DCCD. (C) Absorbance change induced by a single flash after a period of 60-sec illumination, in the presence (△) and absence (●) of 5×10^{-8} mol DCCD. All measurements were performed in a medium containing chlorophyll 75 μg/3 ml, Hepes-KOH 20 mM (pH 7.5), sorbitol 330 mM, MgAc 2 mM, KH_2PO_4 2 mM, DTE 2 mM, and ATP 10^{-5} M.

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 (Peters *et al.*, 1983). From these experiments it was concluded that energization of the thylakoid membrane results in the specific suppression of the reaction 2 component in the light-induced P515 response.

Obviously, the acceleration of the overall decay rate of the P515 response generally found after short periods of illumination of chloroplasts (Morita *et al.*, 1981; Witt and Moraw, 1959) and green algae (Joliot and Delosme, 1974) 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. 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 decreased from 40 μmol ATP/mg chl./hr in the absence of DCCD to 1.4 μmol ATP/mg chl./hr in the presence of 5×10^{-8} mol DCCD. 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. 1A). Obviously, at this low concentration DCCD does not cause inhibition of electron transfer as reported by Uribe (1971) for higher concentrations. As can be seen from Fig. 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.

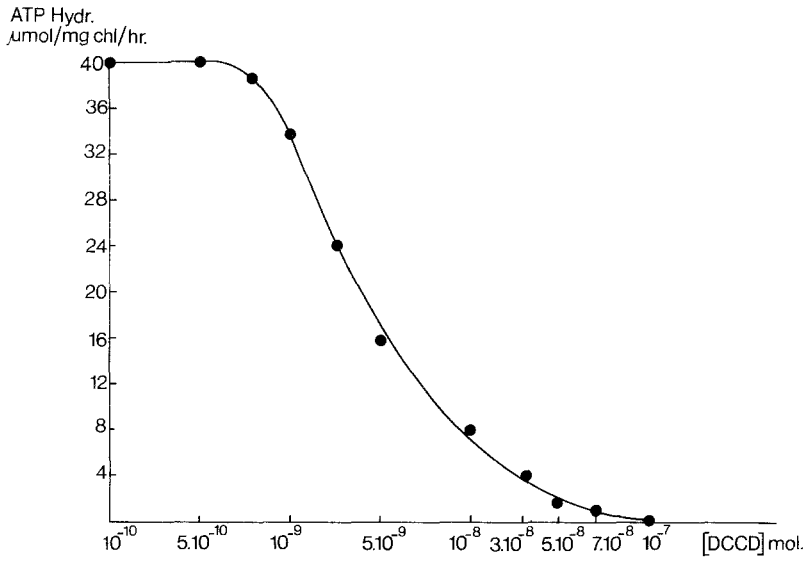

Fig. 2. Rate of ATP hydrolysis measured in broken chloroplasts after a period of 60-sec illumination as a function of the concentration of DCCD. The measurement was performed in medium (volume 3 ml) containing chlorophyll 75 μm/3 ml, Hepes-KOH 20 mM (pH 7.5), sorbitol 330 mM, MgAc 2 mM, KH_2PO_4 2 mM,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

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. 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 is shown to be a very effective concentration for the inhibition of the ATPase (see Fig. 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^+ 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. Accordingly, the suggestion made by others (Joliot and Delosme, 1974; Morita *et al.*, 1981; Witt and Moraw, 1959; Girault and Galmiche, 1978; Junge *et al.*, 1970) 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

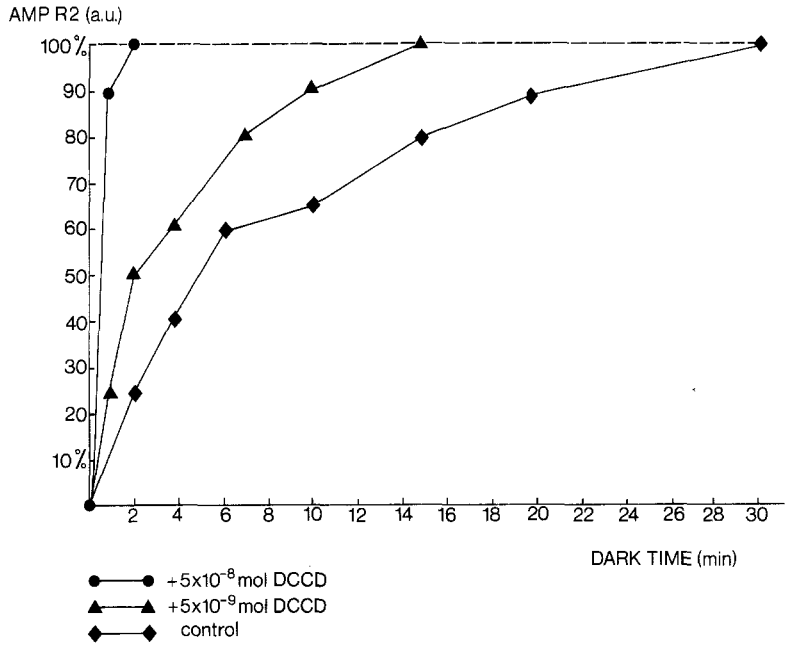

Fig. 3. The effect of dark adaptation after a 60-sec preillumination on the recovery of the amplitude of reaction 2 in chloroplasts in the absence (♦) and presence of 5×10^{-9} (▲), or 5×10^{-8} mol DCCD (●). The measurement was performed in a medium containing chlorophyl 75 μg/3 ml, Hepes-KOH 20 mM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

light-driven electron transport or by reverse electronflow caused by ATP hydrolysis in the dark. Even in the presence of 5×10^{-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. 3, the suppression of reaction 2 by a period of 60 sec illumination of broken

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.[a]

Number of moles 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

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

chloroplasts in the presence of DTE and ATP is diminished from 30 min in control chloroplasts to 2 min in the presence of 5×10^{-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). This finding is important for the interpretation of the experimental results reported by Morita *et al.* (1982). 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. 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 adapation, 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 *et al.* (1982) 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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