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The effect of uncouplers (F)CCCP and NH_4Cl on the kinetics of the flash-induced P515 electrochromic bandshift in spinach chloroplasts

R.L.A. Peters, O. van Kooten and W.J. Vredenburg

Laboratory of Plant Physiological Research, Argricultural University, Gen. Foulkesweg 72, 6703 BW Wageningen, The Netherlands

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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.

P515 *Electrochromic bandshift* *Uncoupler* *FCCP* *CCCP* *NH_4Cl*

1. INTRODUCTION

According to [1], the multi-phasic kinetic pattern observed in the P515 electrochromic bandshift induced by a saturating single turnover light flash in dark-adapted chloroplasts, is the composite result of at least two different reactions, called reactions 1 and 2. In their interpretation, reaction 1, characterized by a fast rise (ns) and a subsequent single exponential dark decay with a rate constant of the order of 10 s^{-1}, is the reflection of the generation and decay respectively, of a transmembrane delocalized electric field induced by the light-induced charge separation in PS I and PS II.

Abbreviations: P515, absorbance change at 518 nm; PS, photosystem; R1, reaction 1; R2, reaction 2; Chl, chlorophyll; Fe-S, Rieske iron-sulphur protein; cytb-f, cytochrome *b*-563-cytochrome *f* complex; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ATP, adenosine 5'-triphosphate; BSA, bovine serum albumin

Reaction 2, however, characterized by a relative slow increase in absorbance during the first 100–150 ms after the flash and a decay with a first order rate constant of about 1 s^{-1} (i.e., a morefold of the rate constant of the decay of reaction 1) is thought to be related to intramembranal electrical phenomena [1–3], presumably associated with the liberation of protons in inner-membrane domains near the cytb-f protein complex [4]. 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 [5] is largely reduced in chloroplasts isolated from plants which were grown at low light intensity. It was found 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 [5]. Here, we present results in support of our suggestion that the reaction 2 component of the

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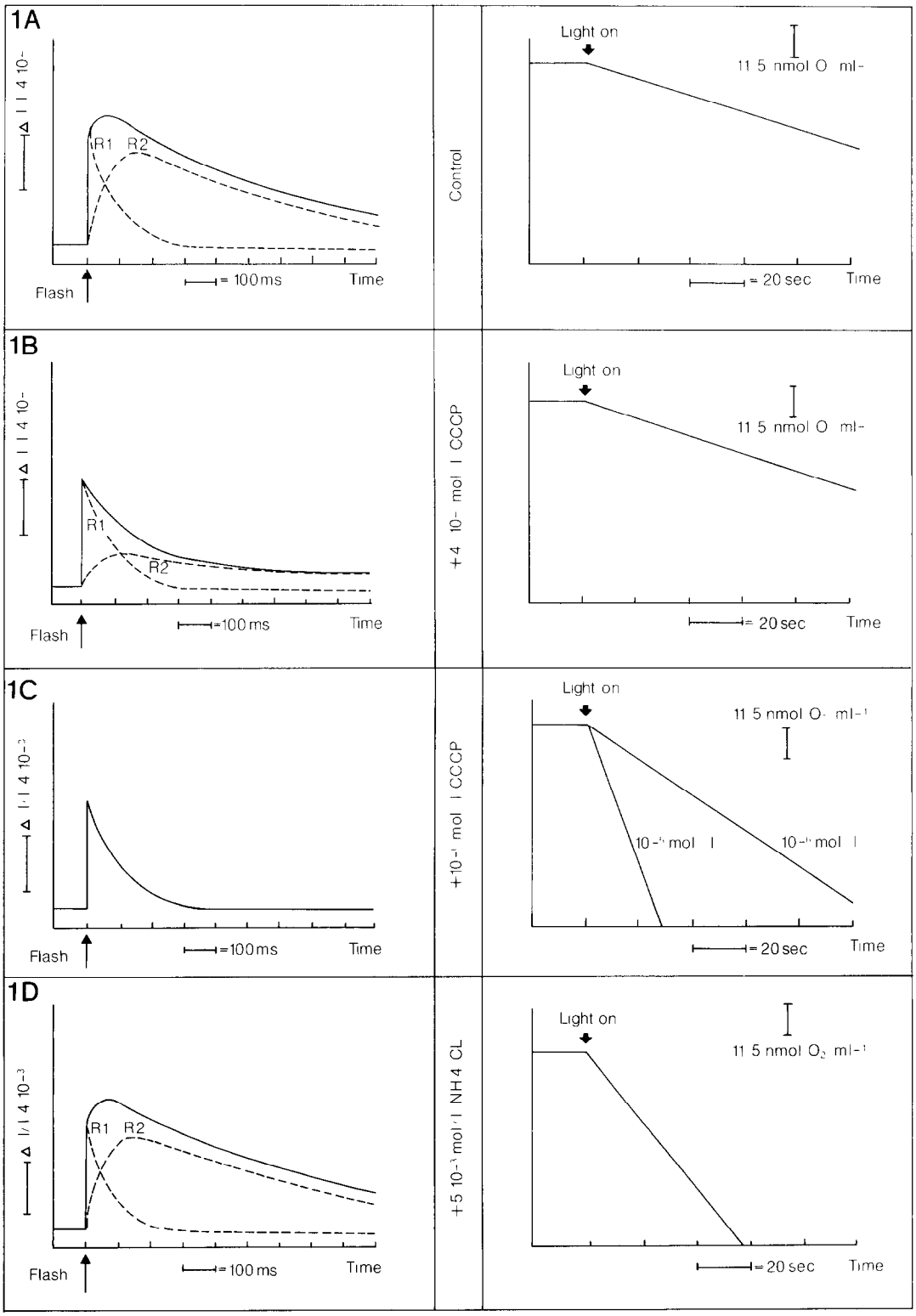
1A
R1
R2
Flash
= 100 ms
Time
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Light on
11 5 nmol O ml-
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Time
1B
R1
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Time
+4 10- mol l CCCP
Light on
11 5 nmol O ml-
= 20 sec
Time
1C
Flash
=100 ms
Time
+10-4 mol l CCCP
Light on
11 5 nmol O- ml-1
10-5 mol l
10-6 mol l
= 20 sec
Time
1D
R1
R2
Flash
= 100 ms
Time
+5 10-3 mol l NH4 CL
Light on
11 5 nmol O2 ml-1
= 20 sec
Time

flash-induced P515 response 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 5×10^{-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.

2. 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 approx. 100 $W \cdot m^{-2}$ 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 [6] as described in [7]. This procedure routinely yielded preparations with 90–95% intact chloroplasts as determined by ferricyanide reduction [8]. 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 [9]. 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 2×10^{-5} M methyl viologen as an electron acceptor. The oxygen consumption was measured with a Gilson oxygraph as in [10].

3. RESULTS AND DISCUSSION

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.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 [1,9] 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.1B. From this it can be seen that the contribution of

←

Fig.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 4×10^{-7} M and 10^{-6} M CCCP (b, c, respectively) and 5×10^{-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).

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.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. 2, progressive addition of CCCP to a sample of intact chloroplasts in the range 10^{-8}–10^{-6} M results in the selective suppresion 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. 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.1D and fig.3, the addition of 5×10^{-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. 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

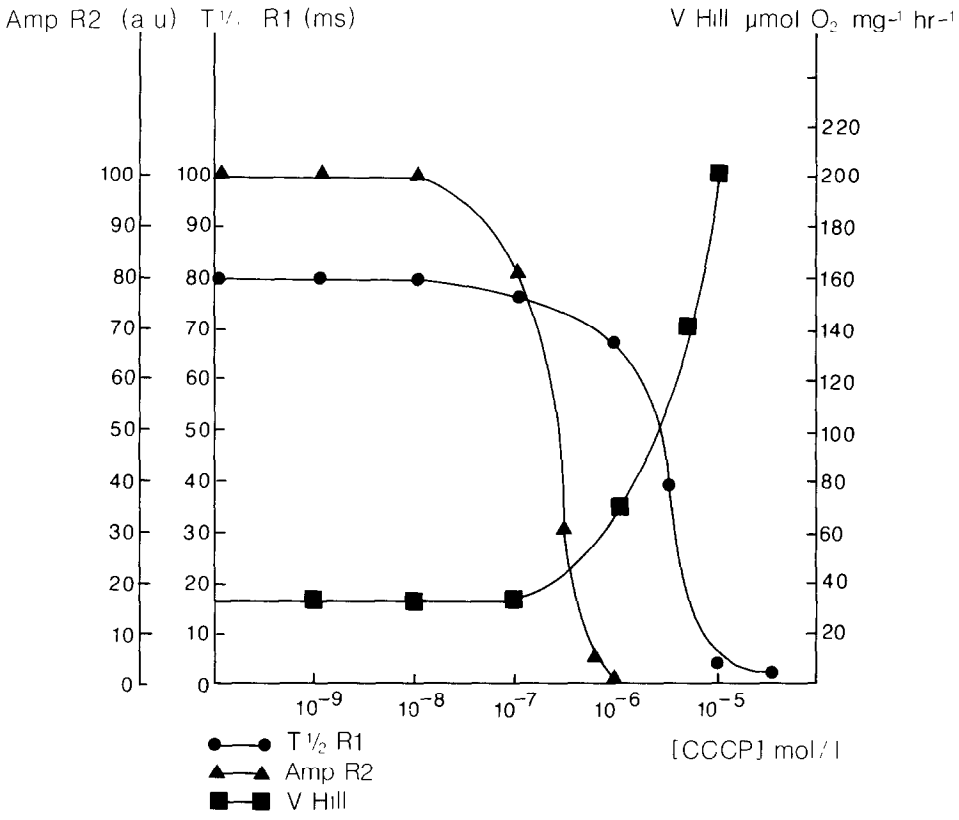

Fig.2. The amplitude of reaction 2 (▲—▲) the half-lifetime of reaction 1 (●—●) and the Hill reaction rate (■—■) in intact chloroplasts as a function of CCCP concentration.

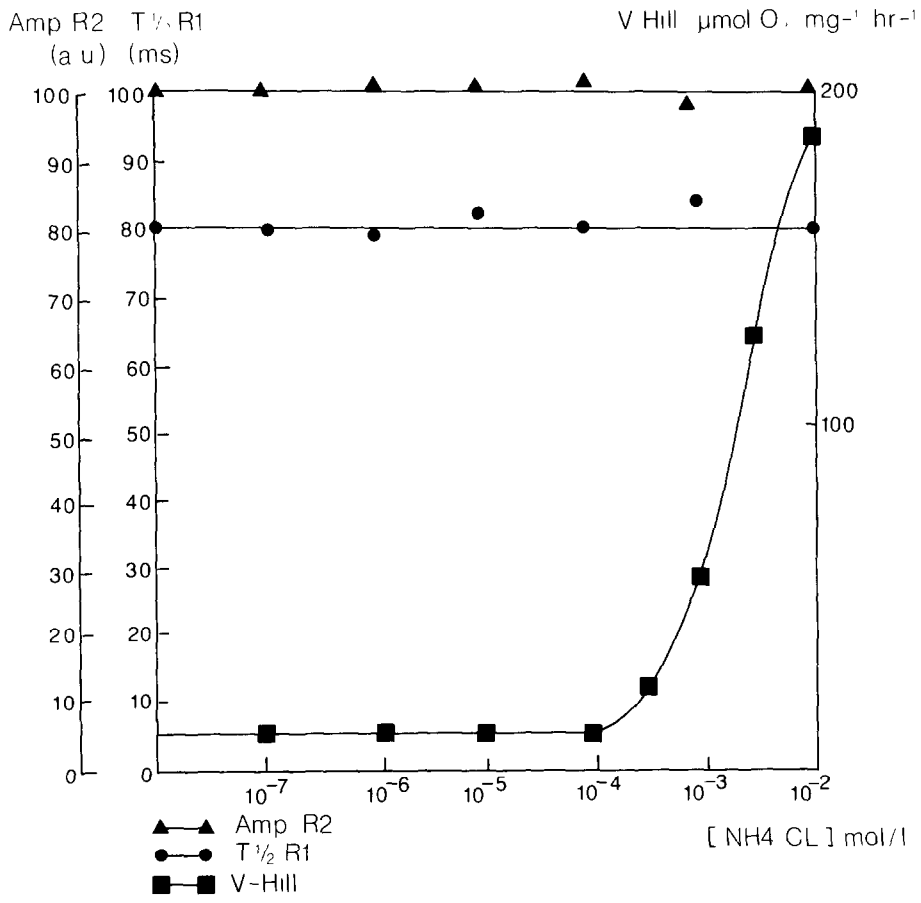

Fig.3. The amplitude of reaction 2 (▲—▲), the half-lifetime of reaction 1 (●—●) and the Hill reaction rate (■—■) in intact chloroplasts as a function of NH_4Cl concentration.

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 [4] 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 synthetase). Models depicting site specific intramembranal proton processing in the thylakoid have been suggested [11,12]. In this respect it is of interest to mention that, in conformation with results of others [2,3], we have shown that reaction 2 can also be induced in the dark towards its saturation level by ATP-driven electron flow (proton translocation) [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 we have shown [5] 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 $cytb_{563}$ and cytf [5].

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 5×10^{-3} M 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. Part of these studies, notably those focussing on the kinetics of light-induced changes in inner thylakoid proton deposition in relation to reaction 2 and ATP synthesis, are presently being carried out and will be dealt with in a forthcoming paper.

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