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The flash-induced P515 shift in relation to ATPase activity in chloroplasts

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The flash-induced P515 signal shows complex kinetics in its rise and decay. The fast rising component, reaction 1/RC, is attributed to the transmembrane charge separation in the reaction centra of Photosystem I and II. The slow component consists of a fast-decaying electrogenic part, reaction 1/Q (due to a Q-cycle) and a slow decaying component, reaction 2. In this work we examined the relation between the onset of flash-induced ATP synthesis, the steady-state ATP synthesis rate and the reaction kinetics of the distinguishable P515 components. The following results have been achieved. (i) The overall P515 decay, if not corrected for reaction 2, is an inadequate measure for the ATPase activation state. (ii) Only the dissipation of the transmembrane electric field, as sensed by reaction 1/RC, is stoichiometrically accelerated by a CF_0 proton conductance which, under ATP synthesizing conditions, is added to the actual passive membrane conductance. (iii) ATP hydrolysis does not result in an acceleration of the decay of reaction 1/RC. (iv) The decay of reaction 2 is not altered during activation of the ATPase.

Introduction

It is generally recognized that the activation of the ATPase has a great influence on the decay kinetics of the P515 signal [1–3]. In recent literature [4,5] the overall decay rate of the P515 signal has been taken as a direct measure of the activation state of the thylakoid ATPase. However, the slow-rising component in the P515 absorbance change, showing multiphasic rise and decay kinetics, is the subject of different interpretations. Generally the slow rise is attributed to an additional transmembrane charge separation due to an electrogenic Q-cycle [6–8]. In contrast we recently showed the slow rising phase in fully dark-adapted chloroplasts to consist of two kinetically different components [9]. One component is electrogenic and due to an active Q-cycle. The other component, called reaction 2, is different in the sense that its decay is significantly slower than the decay associated with the transmembrane primary charge separation. This makes this component unlikely to be transmembrane electrogenic. It has been suggested to be related with innermembrane proton domains [10–12], possibly connected with the ATPase. Reaction 2 can be saturated, either by a few pre-flashes, a weak pre-illumination or by ATP hydrolysis [9–11]. After a few flashes, the P515 signal is enriched in reaction 1/RC which is superimposed upon the saturated reaction 2 absorbance level, resulting in an apparent strong enhancement of the overall decay of the flash-induced P515 signal.

The apparent accelerated decay of the P515 signal under phosphorylating conditions is generally ascribed to the increased proton conductivity through the ATPase. The decay of the overall P515 signal has been taken as a measure of the activation state of the ATPase. However, in none of these studies [2,4,5,13] has the slow decaying component (reaction 2) been taken into account. This may lead to serious misinterpretation of the decay kinetics of the flash-induced P515 signal and the activation state of the ATPase.

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

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, dicyclohexylcarbodiimide; DTE, dithiothreitol; P515, absorbance change at 518 nm; pmf, protonmotive force; ttx, tentoxin.

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standing of the relation between the P515 decay kinetics, the onset of ATP synthesis and the steady-state ATP synthesis rate.

Materials and Methods

Isolation of pea chloroplasts

In all experiments we used freshly prepared pea chloroplasts. Pea plants were grown at a light intensity of 33 W/m^2, a temperature of 20 °C and a relative humidity of 70%. Chloroplasts were isolated from 2-week-old seedlings. Isolation was done under weak light, maximum intensity 0.1 W/m^2. Freshly harvested leaves were mixed in a Sorvall Omnimixer three times for 1 s at maximum speed in isolation medium, which contained: 343 mM sorbitol, 0.4 mM KCl, 0.1 mM $MgCl_2$, 2 mM ascorbate, 4 mM cysteine and 50 mM Hepes/KOH (pH 7.8). The suspension was filtered through three layers of nylon cloth and centrifugated for 50 s at 1000 × *g*. The pellet was washed in resuspension medium containing: 343 mM sorbitol, 5 mM KCl, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM KH_2PO_4, 2 mM ascorbate, 4 mM cysteine, 10 mg/ml BSA and 50 mM Hepes/KOH (pH 7.8). Finally the chloroplasts were resuspended in a small volume of resuspension medium and stored on ice in the dark.

Flash-induced ATP formation

Flash-induced ATP formation was measured by the luciferin-luciferase luminescence detection method, using the LKB WALLAC ATP monitoring kit [14]. The contents of one vial of LKB ATP monitoring reagent is diluted in 10 ml distilled water and stored in 1 ml portions at −18 °C. For each experiment chloroplasts were broken prior to the experiment in the cuvette. We used two different reaction media. Medium A containing: 10 mM sorbitol, 3 mM $MgCl_2$, 1 mM KH_2PO_4 and 50 mM Tricine/KOH (pH 8.0) is normally used in experiments monitoring ATP formation [14]. Medium B contains: 330 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 75 mM Hepes/KOH (pH 7.5) and is one of the standard media in P515 experiments [9]. Further additions used in medium A and medium B were: 5 mM dithiothreitol, 5 μM diadenosine pentaphosphate, 0.1 mM methyl viologen, 0.1 mM ADP, 200 μl ATP-monitoring kit and chloroplasts equivalent to 25 μg/ml chlorophyll. During the measurement the temperature was kept at 10 °C and the suspension was gently stirred. Actinic flashes were guided to the cuvette by a light fiber shielded by a Schott RG 645 filter. The photomultiplier was protected from actinic light by a Balzer K-55 and a Corning 4-76 filter combination. Changes in bioluminescence at 563 nm were recorded on a pen recorder. After each measurement the system was calibrated by adding 20 nmol ATP from a stock solution to the reaction medium.

Flash-induced absorbance changes

P515 measurements were carried out using a modified Aminco Chance spectrophotometer [15]. Signal processing was as described by Snel [15]. For all measurements chloroplasts were dark-adapted for at least 2 h. All signals were recorded as single sweeps, without averaging, at a temperature of 10 °C. P515 measurements were carried out in the same medium as ATP measurements, either in medium A or B, in which the ATP monitoring kit is replaced by reaction medium.

P515 curves were fitted as the sum of exponential functions, using the weighted least squares fitting procedure, yielding the best-fit parameters [16]. All fits are corrected for the non-electrochromic gramicidin insensitive component, reaction 3 [20], which contributes to about 5% of the total amplitude and decays very slowly (1–5 s).

Results

Flash-induced ATPase activity

Fig. 1 shows two typical measurements of flash-induced ATP formation. The spikes on top of the signal are due to flash artifacts and can be used as flash markers. The measurement shown in trace A, is performed in medium A. The lower trace (B) shows a measurement performed in medium B, usually used for

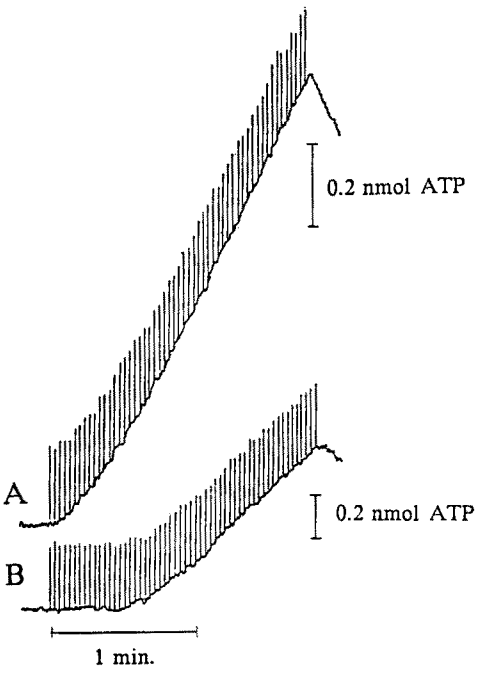

Fig. 1. Time recordings of flash-induced ATP formation as measured by the luciferin-luciferase luminescence detection method. Curves A and B measured in reaction medium A and B (see text), respectively. All further conditions as given in Material and Methods. Flash frequency 0.5 Hz. The vertical spikes on the signal mark the time points of flash firing. Steady state flash yield 0.53 and 0.46 nmol ATP/flash per mg Chl for A and B, respectively. Note difference in ATP calibration bars.

P515 measurements. The number of flashes given at the point where the first rise is detectable is taken as the minimal number of flashes required to initiate ATP formation. We will term this the 'Onset flash lag'. The ATP flash yield is determined from the slope of the signal where a steady rise is reached.

It is shown that in medium B the onset of ATP formation is substantially delayed. We studied the number of flashes needed to initiate ATP synthesis in relation to the flash frequency. The number of flashes required for the onset of ATP synthesis, the onset flash lag, in medium B is about 18 and is independent of the flash frequency within the range of 0.2–5.0 Hz (Fig. 2). In contrast, the steady-state ATP production is decreased by lowering the flash frequency (Fig. 2). This experiment was repeated using the reaction medium A and gave qualitatively the same results. The number of flashes required for the onset of ATP formation did not exceed three flashes and was also found to be independent of the flash frequency. The steady-state ATP yield per flash was found to decrease at lower frequency similarly as in medium B.

Fig. 3 shows the P515 signals in response of the first flash in medium A and B, respectively. Fast- and slow-rising components, different in proportion, are clearly distinguishable in both responses. In medium B the overall decay of the P515 signal is accelerated. The fast rise in the P515 signal, equal in both media, is attributed to the transmembrane primary charge separation, reaction 1/RC [9]. The slow component associated with the Q-cycle (reaction 1/Q), can be neglected here because, under the experimental conditions used, its contribution is only small and consequently does not significantly influence the overall P515 signal [9]. As has been shown before [9,10] and as illustrated in Fig. 7 (see below), the slow rising component reaction 2 can be saturated by a few shortly spaced flashes. Thus, upon

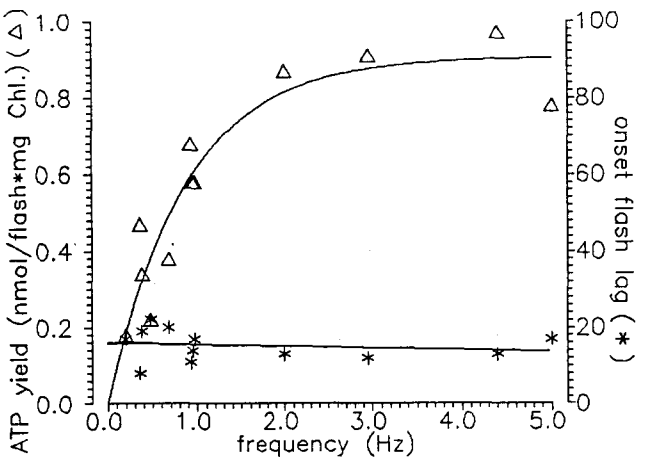

Fig. 2. Flash frequency dependency of ATP yield and onset flash lag (see text). Measurements performed in reaction medium B, further additions as indicated in Material and Methods.

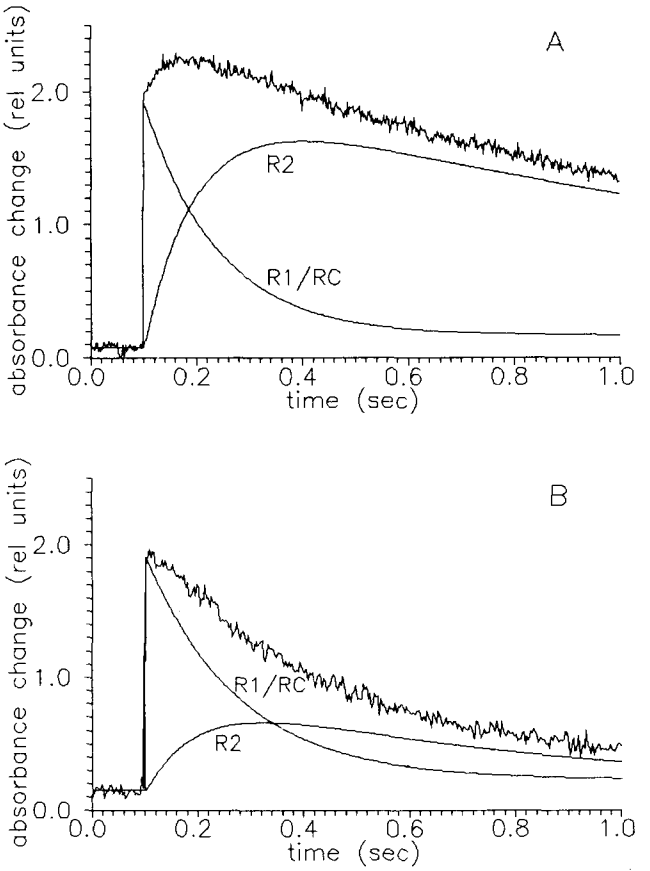

Fig. 3. Flash-induced P515 signal upon a single first flash, fired at $t = 0.1$ s, in dark-adapted chloroplasts. Measurement in reaction medium A and B, respectively. R1/RC and R2, fitted curves for reaction 1/RC and reaction 2, respectively. See text for further details.

following flashes the initial decay of the overall response is not mixed with a slow rising phase of reaction 2 any more, and is exclusively determined by the decay of reaction 1/RC. Consequently, after a few shortly spaced saturating flashes the kinetics of reaction 1/RC can be determined by analysis of the decay of the P515 signal. This analysis was performed in separate experiments for both media. The decay constants for reaction 1/RC were 7.2 and 5.8 in media A and B, respectively (curves 1/RC in Fig. 3A and B). Consequently the great difference in the overall P515 signal in both media is due to a substantially lower extent of the slow component, reaction 2, in medium B. This lower extent of reaction 2 coincides with a longer onset flash lag in medium B (Fig. 1).

The effect of CCCP

Addition of low concentrations of CCCP, up to 0.4 μM, increases the onset flash lag, but has no influence on the steady-state ATP production (Table I). Lowering the flash frequency in the presence of CCCP reveals that the onset flash lag is no longer frequency-independent (Fig. 4). The steady-state flash-induced ATP pro-

TABLE I

Effect of addition of CCCP on the onset flash lag and the steady-state ATP yield

Measurements performed in reaction medium A, with further additions as indicated in Material and Methods. Flash frequency, 1 Hz.

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

duction in the presence of these concentrations of CCCP is frequency dependent and shows the same frequency profile as the control (Fig. 4, Table I). In addition, CCCP at the concentrations used strongly inhibits the slow component reaction 2 in the P515 signal, without affecting the decay of the transmembrane component reaction 1/RC (Fig. 5) [25].

Recently is was shown possible to manipulate the coupling mode (localized or delocalized) between the electron transport and ATP synthesis by changing the salt concentrations in the chloroplast storage medium [14,17]. We repeated these experiments, and measured the onset flash lag of chloroplasts, stored either in a low salt medium (200 mM sorbitol) or in high-salt medium (100 mM KCl), in reaction medium A. We found an onset flash lag of 4 and 17 flashes in low- (200 mM sorbitol) and high- (100 mM KCl) salt stored chloroplasts, respectively (not shown). The P515 signals measured under the same conditions revealed a suppression of reaction 2 in high-salt stored chloroplasts (not shown, but see Figs. 3B and 5B for comparable signals). Thus, the effect of storage in high-salt medium is very much

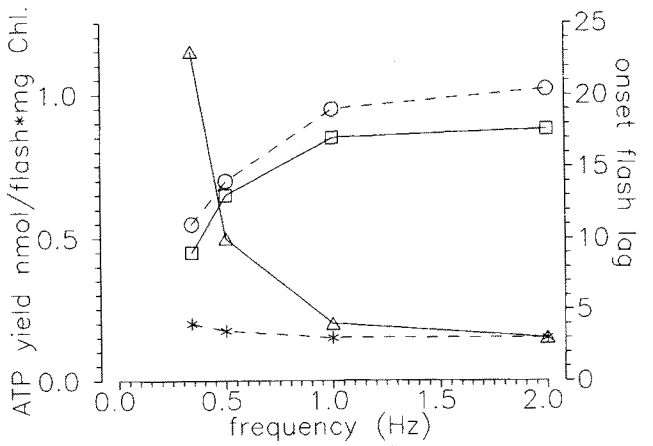

Fig. 4. The ATP yield (○, □) and the onset flash lag (△, *) as a function of the flash frequency in the absence (○, *) and presence (△, □) of 0.3 μM CCCP, respectively. Measurements performed in assay medium A, further additions as indicated in Material and Methods.

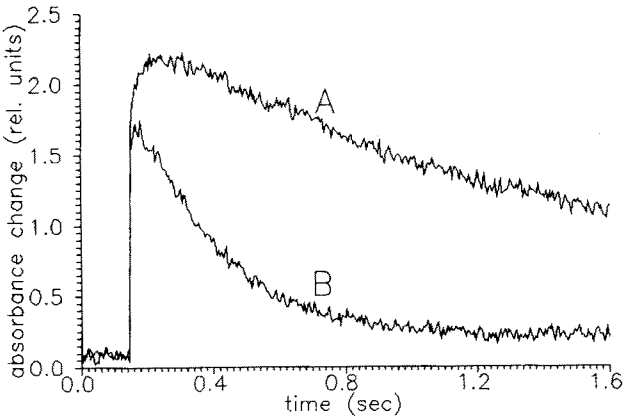

Fig. 5. Flash-induced P515 signal in dark-adapted chloroplasts. Measurement in reaction medium A, with additions as indicated in Material and Methods. Curve A, control; curve B, in the presence of 0.3 μM CCCP.

the same as addition of low concentrations of CCCP to the chloroplast preparation as used in our experiments (Table I and Fig. 5).

The P515 signal during steady-state ATP production

After about 30–40 flashes in a 1 Hz flash train, the ATP synthesis reached a constant rate (Fig. 1). We suppose that in this situation the ATPase has reached its maximal activation state under the experimental conditions used. In parallel experiments we measured the developing ATPase activity in a 1 Hz flash train and the P515 signals in the 60th flash. Fig. 6 shows the single exponential fits of the initial fast P515 component upon the 60th flash, obtained after correction for the (slow) decay of the reaction 2 component saturated

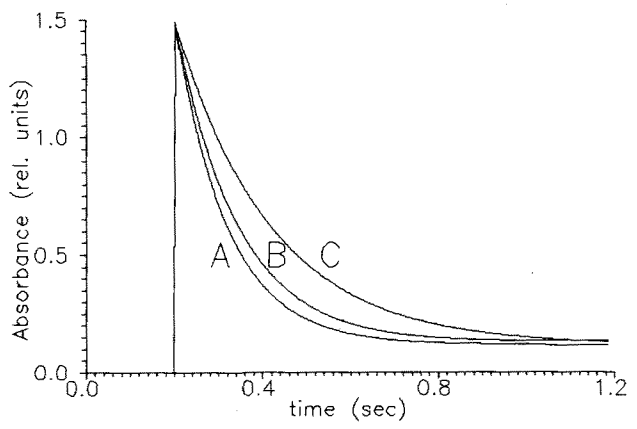

Fig. 6. Single exponential fits of flash-induced P515 responses upon the 60th flash of a 1 Hz flash train, after correction for the slow-rising reaction 2 (see text). Measurements during ATP synthesis in the presence of ADP and DTE in reaction medium B, further additions as indicated in Material and Methods. Curve A, in the absence, curve B in the presence of 25 μM DCCD, respectively. Steady-state ATP synthesis for B was 60% of that of control A. Curve A and B are averaged fits of three separate experiments. Curve C, reaction 1/RC as determined from a separate multi-flash experiment in the absence of ADP and DTE (see text for further details).

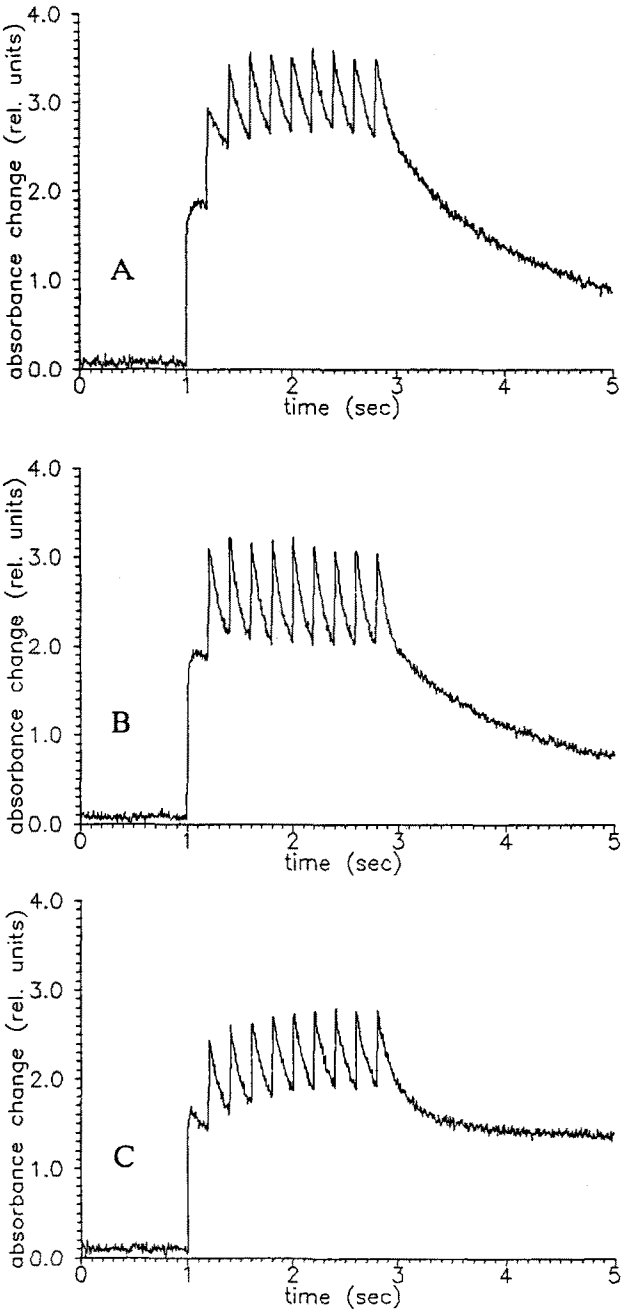

Fig. 7. Multi-flash P515 signals measured in reaction medium A. Additions as indicated in Material and Methods. Curve A, control in the absence of DTE and ADP. Curve B, with addition of DTE and ADP. Curve C, with addition of DTE and ATP.

in the foregoing flashes (see for the overall decay in a similar experiment but after 10 flashes, Fig. 7). Curve 6A shows the signal in the presence of ADP, curve 6B in the presence of ADP and 25 μM DCCD. Curve C represents the response as determined from an independent experiment using multiple flashes at 5 Hz frequency in the absence of DTE and ADP [10] (see also Fig. 7A for an illustration). At the concentration of DCCD used here [18], the ATP synthesis rate was determined to be 60% of the control value. Unfortunately, it was not possible to use higher concentrations of DCCD, to obtain a stronger inhibition of the ATPase. We found higher DCCD concentrations to cause an acceleration of the P515 signal under control conditions, i.e., in the absence of ADP and DTE [19]. Because of the correction for the contribution of reaction 2, the curves in Fig. 6 are those of reaction 1/RC, and represent exclusively the rise and decay of the transmembrane electric field. Two pathways for back-flow of the transmembrane charge are generally recognized [34,35]; the passive dissipation through the membrane and secondly the proton conduction through the ATPase. This leads to the following equation for the time pattern of the electrogenic P515 signal:

$$A(t) = A(0) \cdot \exp\left[-(k_1 + k_{atp}) \cdot t\right] \quad (1)$$

where $A(0)$ is the amplitude at $t = 0$, k_1 and k_{atp} are determined by the passive membrane leakage and the proton conductivity of the membrane ATPase, respectively. The sum of k_1 and k_{atp} represents the total decay constant. We assume k_1 to be independent of the ATPase activity. We further assume the proton conductivity through the ATPase to be linearly related to the ATP production rate. Thus, according to the ATP measurements k_{atp} in curve B is 60% of its maximum value in curve A. Estimation of the value of the total decay constants $(k_1 + k_{atp})$ from the curves shown in Fig. 6 then leads to the calculated values of k_1 and k_{atp} as shown in Table II.

The decay constant of reaction 1/RC, determined in a separate experiment under conditions where no ATPase activity is detected (curve C), is in good agreement with the value for k_1, as calculated from curves A and B. This experiment reconfirms the transmembrane charge dissipation to be accelerated by an enhanced proton conductivity associated with ATP synthesis. The experiment is essentially similar, except for corrections of the slow components, to the classical experiment of Junge et al. [35] showing an enhanced P515 decay in the presence of ADP and P_i. Taken together, this supports

TABLE II

Decay constants for the curves shown in Fig. 6

The total decay constant $(k_1 + k_{atp})$ for curve A and B are averaged from three separate experiments. k_1 and k_{atp} are calculated using the assumptions mentioned in text. k_1 is determined by passive membrane leakage. k_{atp} is attributed to proton conductance through the ATPase.

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

TABLE III

Fit parameters of exponentials fits from P515 signals measured under conditions as in Fig. 7

Fits were made from the time point of the tenth flash. Al and A2 amplitude of reaction 1/RC and reaction 2, respectively. k_1 (s^{-1}) and k_{atp} (s^{-1}) as in Table II. k_2 (s^{-1}): decay constant of reaction 2. Parameters of the 'control' and '+ADP' are averaged from five separate experiments. All other parameters are determined from a single experiment. Concentration tentoxin (ttx) 10 μM.

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

our view that reaction 1/RC exclusively monitors the transmembrane electric field.

The P515 signal during flash ATPase activation

We further analyzed the P515 signal in medium A under different conditions in repetitive flash experiments. Fig. 7 shows the P515 signal under control conditions (A), in the presence of 0.1 mM ADP (B) and in the presence of 0.15 mM ATP (C), respectively. The amplitude and decay of the P515 signal in the control measurement and in the presence of ADP after the first flash are equal. However, after the second flash the decay of the signal is accelerated in the presence of ADP (Fig. 7B). This coincides with the onset of ATP production in the second or third flash under the conditions used.

The P515 signal in the presence of ATP shows already in the first flash an accelerated decay relative to the control experiment. After the tenth flash, the signal measured in the presence of ATP does not decay towards the original zero level, but remains on a higher level. Analysis of the decay kinetics after the tenth flash gives the results shown in Table III. It illustrates the amplitude of reaction 1/RC to be nearly constant under the different conditions. The decay of reaction 1/RC is accelerated in the presence of ADP. In the presence of 0.15 mM ATP, when hydrolysis takes place, the total decay constant ($k_1 + k_{atp}$) is not significantly different from the control measurement. Obviously, the expected enhanced proton conductivity, due to the activation state of the ATPase, does not result in accelerated decay of reaction 1/RC. Probably the enhanced proton conductivity is compensated by a continuous proton influx resulting from ATP hydrolysis. Thus under these conditions, the decay of reaction 1/RC is solely determined by the passive leak through the membrane. Table III (line 4) shows the results of an experiment conducted under the same conditions as in Fig. 7. The decay of reaction 1/RC in the presence of the CF_1 antagonist tentoxin is inhibited compared to the decay under fully activated conditions (compare line 2 and 4 in Table III). If we adopt the assumptions made for the calculation on the DCCD inhibition in Fig. 6, the results in Table III reveal a 72% inhibition of the ATPase. This result is in good agreement with the inhibition of the ATP synthesis rate of 79% actually measured.

Although there is a tendency to a lower amplitude of reaction 2 in the presence of ADP relative to its amplitude in the absence of ADP, this has not been definitely established (Table III). In the presence of ATP, the amplitude of reaction 2 is lower and its decay is substantially inhibited. Schreiber and Rienits [12] showed that dark addition of ATP to a chloroplast suspension with pre-activated ATPase results in a dark absorbance change at 515 nm to a stationary level. The result shown in Fig. 7C is in excellent agreement with these observations and illustrates the slow component in the P515 signal to be activated and saturable by ATP hydrolysis.

Discussion

It is known that the activation of the ATPase as well as the steady-state ATP synthesis needs membrane energization [21]. The differential dependency of the onset flash lag and the steady-state ATP flash yield on the frequency suggest different mechanisms for the accumulation of flash-induced protonmotive force (pmf). Obviously, the dark period between flashes has no influence on the onset flash lag, indicating the absence of any appreciable dark dissipation of energy required for the activation of the ATPase. In contrast, the flash frequency dependency of the steady-state ATP flash yield suggests a considerable leak of energy required for ATP synthesis in the dark period between flashes.

From P515 measurements (Fig. 7, Table III) it is clear that the transmembrane electric field, sensed by reaction 1/RC, decays with a half-time of about 110 ms. Thus, in the frequency range 0.2–5 Hz it seems inappropriate to expect the transmembrane electric potential to be responsible for the flash frequency independency of the onset flash lag. Using attenuated flashes in a system where the electric component of the pmf is eliminated by the addition of nonactin, similar results were found [22]. It seems reasonable to conclude that a stable proton gradient is responsible for the flash frequency independency of the onset flash lag. Several authors reported on the existence and function of proton domains which are not in equilibrium with the bulk aqueous phase involved in the activation of the ATPase [14,17,23,24].

As shown in Table I, low concentrations of CCCP increased the onset flash lag. Moreover, in the presence of CCCP the onset flash lag is no longer frequency-independent. However, CCCP concentrations up to 0.4

μM did not decrease the ATP flash yield during steady-state (Table I and Fig. 4), but caused an increase in decay of the overall P515 signal (Fig. 5). In the experiment presented in Table I, we used a flash frequency of 1 Hz, whereas the maximum flash yield is reached only at about 2 Hz (Fig. 2). We expect the lower ATP yield at lower flash frequencies to be due to passive membrane leaks. If CCCP induces a membrane leak competing with the ATPase for protons, one would expect a decreased ATP yield per flash in the presence of CCCP. Thus the apparent acceleration of the P515 signal by low concentrations of CCCP is most probably due to the inhibition of the slow component, reaction 2, and is not caused by an enhanced transmembrane proton conductivity.

Under phosphorylating as well as under non-phosphorylating conditions, the initial decay of the flash-induced P515 signal seems to be accelerated after a few shortly spaced flashes (Fig. 7). In our interpretation, the apparent accelerated decay is due to the saturation of reaction 2 [9–11]. Consequently, the slow rise of reaction 2 does not contribute to the initial decay of the P515 signal (cf. Fig. 7), and the curve fitting can be done properly without further assumptions. The experiments shown in Figs. 6 and 7 and Table II and III demonstrate the decay constant of reaction 1/RC to be related to the activation state of the ATPase. It is also demonstrated that the decay of reaction 2 is not influenced by the activation state of the ATPase. Consequently, it should be emphasized that the studies of Wise and Ort [4] and Kramer and Crofts [5], relating the decay of the overall P515 signal to the ATPase activation state, might be in error. In these studies the contribution of the slow reaction 2 component, which is explicitly present in dark-adapted intact plant material, has not been taken into account.

Conspicuous is the almost fully inhibited decay of reaction 2 in the presence of ATP (Fig. 7 and Table III). This is in full agreement with earlier results [26]. Schreiber and Rienits [12] showed an ATP-induced dark absorbance change at 515 nm in chloroplast suspensions with an activated ATPase. This absorbance change coincides with a complementary loss of light-induced absorbance change, and was attributed to the saturation of reaction 2 [12]. Fig. 7C illustrates the saturation of reaction 2 by ATP hydrolysis inducing a stationary higher absorbance level.

The presence of ATP and DTE can lead to autocatalytic activation of the ATPase [27]. This activation is possibly intensified by the measuring beam. A slightly higher intensity of the measuring beam was indeed found to lead to a lower amplitude of reaction 2 (not shown). This is presumably caused by an enhanced 'zero' absorbance level resulting from partly saturation of reaction 2 due to ATP hydrolysis in the dark. This will complicate the interpretation of the P515 signal in relation to ATPase activity. It needs no argument that signal averaging results in an uncontrolled saturation level of reaction 2 and should not be used in this type of experiment.

Reaction 2

It was earlier suggested that reaction 2 is related to inner-membrane proton domains. This suggestion was based on, amongst other factors, the relatively slow decay kinetics of reaction 2, its saturable character and the specific elimination of reaction 2 by low concentrations of CCCP and valinomycin (see Fig. 5) [9,25,36]. Based on different experimental approaches, other authors have suggested evidence for the existence of proton domains within the membrane [14,23,28–31]. Both proton domains and reaction 2 show a similar sensitivity to ageing and temperature shock [32]. The saturation of reaction 2 by ATP hydrolysis (Fig. 7C) would then suggest the direct release of imported protons in the domain. This would be in agreement with the suggestion that protons released from photosystem II share a common sequestered region with the 8 kDa CF_0 subunit [29,30]. Moreover, in all our experiments, a delayed onset of the ATP synthesis was allied to a smaller extent of reaction 2.

Here we wish to speculate in more detail on the possible relation between reaction 2 and the loading and unloading of proton domains. We found a considerable difference in the ATP onset flash lag and in the extent of reaction 2 in assay medium A and B (Figs. 1 and 3), respectively. The energetic requirements for the onset of ATP synthesis, set by the redox state of the enzyme [21], are unlikely to be different in the two media. Following the possibility of a relationship between inner-membrane proton domains and reaction 2, we expected that in assay medium B (with a high onset flash lag and a decreased extent of reaction 2) the protons would equilibrate with the thylakoid lumen, whereas in medium A, they would not, or at least much less. Beard and Dilley [14] used the effect of the permeable buffer pyridine on the onset flash lag to determine whether proton domains are localized or delocalized. We adopted this method and found no effect of pyridine on the onset flash lag, neither in medium A or in medium B (not shown). Thus, it is likely, in view of the interpretation of Dilley et al. [32], that the proton domains are localized in both media. Furthermore, analysis of P515 signals indicate that the lower extent of reaction 2 in medium B is caused by a somewhat accelerated decay. The accelerated decay in medium B is supposed to be due to enhanced relaxation of a local electric field by other ions than protons. Taking into account the concurrent delayed onset of ATP synthesis in assay medium B, one could conclude that the localized electric field is essential in the onset of ATP synthesis.

Above, we have suggested no primary role for the electric potential in the onset of ATP synthesis. This was based on the finding that the transmembrane electric potential, sensed by reaction 1/RC, decays with a half time of about 110 ms, whereas the onset of ATP formation is frequency-independent in the range of 0.2–5 Hz. In contrast, based on the finding of a delayed onset of ATP formation in the presence of valinomycin and potassium [17,33], the transmembrane electric potential has been reported to be crucial in the onset of ATP synthesis. This seeming discrepancy might be based on an underestimation of an inner-membrane localized electric field. In the presence of valinomycin, not only the transmembrane electric field, but also the local electric field associated with the proton domains and sensed as reaction 2 in the P515 signal, will be abolished [36]. Consequently, it cannot be excluded that, under the conditions used, this local electric field is crucial in the onset of ATP synthesis, instead of the transmembrane electric field.

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