Biochimica et Biophysica Acta, 976 (1989) 28–41
Elsevier

BBABIO 43034

Activation of the chloroplast ATPase measured by the electrochromic change in leaves of intact plants

David M. Kramer and Antony R. Crofts

Department of Physiology and Biophysics, University of Illinois, Urbana, IL (U.S.A.)

(Received 6 December 1988)
(Revised manuscript received 5 April 1989)

Key words: Chloroplast; ATPase; Thiol activation; Electrochromism; Leaf

The activation of the ATPase in intact cucumber leaves has been studied, using a novel instrument developed for the measurement of absorbance changes in intact leaves. The flux through the ATPase was measured by using the decay of the electrochromic shift at 515 nm as an indicator of the flux of protons across the thylakoid membrane. Plots of the rate of decay of the electrochromic shift against its amplitude were used to determine the threshold for activation of the ATPase. A rapid phase of decay of the electrochromic shift was found above a certain threshold amplitude in control leaves. This rapid phase was eliminated after treatment of the leaves with dicyclohexylcarbodiimide, indicating that it was associated with flux through the active ATPase. The threshold amplitude of the electrochromic shift was lower in light-adapted leaves than in dark-adapted leaves, indicating a lower threshold amplitude for the activation of the ATPase. The lowering of the threshold amplitude by light adaptation was eliminated by treatment of the leaves with methyl viologen, which blocks electron flow to the thioredoxin system. These results are interpreted in terms of a model presented by Junesch and Gräber (Biochim. Biophys. Acta 893, 275–288) to explain results from isolated systems. The reoxidation kinetics of the γ-subunit of the ATPase were followed by observing the extent of the slow decay phase of electrochromic shift. These kinetics showed a lag time which was dependent on the amount of light adaptation, and a recovery which did not appear to be dependent on the amount of light adaptation. The kinetics are interpreted in terms of a model consisting of a large redox buffering pool with a midpoint potential somewhat more negative than that of the sulfhydryl groups of the γ-subunit of the ATPase, in equilibrium with a smaller pool equipotential with the γ-subunit sulfhydryls. Some possible characteristics of this pool are discussed. Our results suggest that the activation of the chloroplast ATPase through the thioredoxin system occurs at levels of illumination less than 0.2% of normal actinic intensities, and that this process is never limiting for photosynthesis.

Introduction

In green plants light energy is stored in the form of redox free-energy and a proton gradient generated by light-driven electron transport. The protonmotive force across the thylakoid membrane, in turn, drives the phosphorylation of ADP to ATP catalyzed by the CF_1CF_0-ATP synthase or coupling factor (ATPase). The regulation of the chloroplast ATPase is important, since this enzyme can catalyze not only the synthesis of ATP from ADP and P_i, driven by a pmf across the membrane, but also the reverse reaction, hydrolysis of ATP with the formation of a pmf [1]. Without regulation, the energy stored by poising the ATPase reaction away from equilibrium (ΔG_{ATP}) would be lost when the pmf generated by light-driven electron transport was lower than $\Delta G_{ATP}/n$, where n is the H^+/ATP stoichiometry. The energy stored in ΔG_{ATP} would then be dissipated by coupling of ATP hydrolysis to passive leakage of protons across the thylakoid membrane.

Work with broken chloroplasts and isolated thylakoids has shown that the ATPase is not enzymatically active until a pmf, either as a ΔpH [2–4] or as $\Delta\psi$, or their sum [5,6], is imposed across the thylakoid membrane. The activation by pmf is associated with the release of tightly bound ADP from the CF_1 [7,8], and conformational changes in the ATPase [9]. In addition,

Abbreviations: DCDC, dicyclohexylcarbodiimide; DTT, dithiothreitol; TR, thioredoxin; PS I, Photosystem I; pmf, protonmotive force; P_i, inorganic phosphate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Correspondence: D.M. Kramer, Department of Physiology and Biophysics, University of Illinois, 524 Burrill Hall, 407 S. Goodwin, Urbana, IL 61801, U.S.A.

in the case of green plants, regulation is governed by the redox state of a pair of sulfhydryl groups on the γ-subunit of the ATPase [8–10]. Both the oxidized and reduced forms of the enzyme apparently have the same maximal catalytic rate [13], but the magnitude of the pmf required to activate the reduced form is less than for the oxidized form [13,14]. In in vitro studies, dithiothreitol (DTT) has been used to reduce the sulfhydryls of the γ-subunit, and various oxidants have been used to reoxidize them. It is thought that in vivo the γ-subunit is reduced and oxidized by the thioredoxin (TR) system which, in turn, is reduced by photosynthetic electron flow from PS I through ferredoxin and a ferredoxin : thioredoxin reductase [15–17].

Junesch and Gräber have summarized their studies on isolated systems [13], using a model in which the ATPase can exist in one of four states. Mills and Mitchell [14,18] have presented mathematical simulations of a similar model.

$$\begin{array}{ccc} E_i^0 & \underset{\rightleftharpoons}{\overset{pmf}{=\!=}} & E_a^0 \\ {\scriptstyle TR^-}\ \Downarrow & & \Uparrow\ {\scriptstyle TR^-} \\ E_i^r & \underset{\rightleftharpoons}{\overset{pmf}{=\!=}} & E_a^r \end{array}$$

In the dark-adapted chloroplast, the γ-subunit sulfhydryls are expected to be mostly oxidized and the pmf across the thylakoid membrane is expected to be below the activation threshold. Thus, the ATPase is inactive and this state is termed E_i^0, for the inactive, oxidized enzyme. If a pmf higher than the activation threshold for the oxidized enzyme is imposed across the thylakoid membrane without reducing the γ-subunit sulfhydryls, the ATPase will be in the state E_a^0, active and oxidized. If the γ-subunit sulfhydryls are reduced, by addition of DTT or by the TR system, the ATPase can either be in the E_i^r or E_a^r, for the reduced inactive or the reduced active states, respectively.

The absorbance changes around 515 nm associated with the electrochromic shift have been shown to be linearly related to the electrical potential component of the pmf across the thylakoid membrane (reviewed in Ref. 19). The rate of decay of the electrochromic change should reflect the flux of ions across the membrane down the proton gradient. Under physiological conditions, the major part of the flux reflects the activity of the ATPase, since the proton flux through the ATPase accompanying the synthesis of ATP is the main physiological pathway for dissipation of the pmf. When the ATPase is inactive, the pmf can only decay by slower routes. A correlation between an acceleration of the decay of the light-induced electrochromic shift and the activity of the ATPase isolated from intact leaves has been previously demonstrated [20–22]. The electrochromic shift from dark-adapted leaves, where the ATPase is expected to be oxidized, decayed slowly, and that from light adapted leaves, where the ATPase is expected to be reduced, decayed rapidly, indicating that the light-adapted leaf showed a higher charge flux than the dark-adapted leaf. Similarly, measurements of the ATPase activity of chloroplasts freshly isolated from intact light- or dark-adapted leaves showed a parallel relationship between the rate of decay of the electrochromic shift and the activation of the ATPase.

In this paper, we investigate changes in the activity of the ATPase in intact leaves by measuring the proton flux across the membrane from the decay kinetics of the electrochromic shift. By adjusting the pre-illumination conditions, we can identify each of the four states in the model proposed by Junesch and Gräber [13] by their characteristic decay kinetics. We have employed a new portable kinetic spectrophotometer with a very high signal-to-noise ratio (noise level below 10^{-5} A) [24], allowing measurements in intact leaves of electrochromic changes and cytochrome absorption changes in the time range from microseconds to seconds without signal averaging.

Materials and Methods

Plant growth conditions. Cucumber plants (*Cucumis sativus* L. cv. Ashley) were rised from seed in a soil/peak/vermiculite mixture, watered daily and fertilized weekly. The plants were grown in a controlled environment chamber (600–800 $\mu E \cdot m^{-2} \cdot s^{-1}$, 14 h photoperiod, day/night temperatures of 23 °C/20 °C) as described in Martin and Ort (1985) [23]. Attached leaves which had reached almost full expansion were used for the experiments.

Double-flash kinetic spectrophotometer. The instrument used to measure the kinetics of the electrochromic changes in intact leaves is described in Kramer and Crofts [24]. The design of the spectrophotometer is based on an instrument developed by Joliot and Joliot [25], and achieves very high signal-to-noise ratios (noise levels of less than 10^{-5} A) by utilizing differential optics, and a xenon flash for the measuring beam in place of the conventional continuous measuring beam. This allows the measuring beam to have a higher intensity and thus a higher signal-to-noise level at the discrete time points where measurements are made, while remaining non-actinic over the course of the experiment.

Intact leaves were placed in a special housing, and shielded from stray light by soft foam padding. The atmosphere around the leaf was kept constant throughout an experiment by passing a stream of moistened air into the housing. The instrument could also be used in a portable mode, when run on rechargeable batteries, enabling us to measure electrochromic changes and cytochrome changes in leaves on intact plants in the field.

The degree of saturation of the actinic flashes given by the spectrophotometer was determined by giving a pair of actinic flashes 15 ms apart to a leaf treated with DCMU (100 μM in 0.1% Tween 80 as discussed in the next paragraph), and measuring the extent of the fast (rising) phase of the electrochromic shift at 515 nm after each flash. The fast phase of the electrochromic shift reflects the initial charge separation in open PS II and PS I reaction centers. When dark-adapted DCMU-treated PS II centers receive a photon from the first actinic flash, Q_A becomes reduced and these centers are closed. The electrochromic shift following the second actinic flash can be used to probe the fraction of centers remaining open after the first flash. In all the experiments presented in this paper, the actinic flashes were approx. 50% saturating.

Infusion of reagent into intact leaves. We used the following procedures to introduce reagents into intact leaves. Under dim room light, the upper and low surfaces of the intact leaves were lightly abraded with 400 grit carborundum, and several thicknesses of tissue paper soaked with the reagent solutions were applied to both sides of the leaves. The covered leaves were supported by plastic weighing dishes, and additional small amounts of the reagent solutions were added to the dishes. To aid the infiltration, all solutions included 0.1% Tween 80 as a surfactant. This low concentration had minimal effects on the kinetics of the electrochromic shift, whereas a 10-fold higher concentration increased the decay rate slightly. The reagents were allowed to soak into the leaves in the dark for approx. 30 min.

Results

Effects of light and dark adaptation on the decay of the electrochromic shift

Fig. 1 demonstrates the effects of light and dark adaptation on the decay of the electrochromic shift induced in a cucumber leaf by a single, approximately half-saturating actinic flash. A leaf was dark-adapted for 2 h and then light adapted by illumination with a train of 400 actinic flashes at 20 Hz. During the subsequent dark re-adaptation, at the times indicated (between 10 s and 42 min after the last of the 400 flashes), a single actinic flash was given, and the kinetics of the electrochromic shift were measured at 515 nm. The electrochromic shift decayed much more rapidly in the light-adapted leaf (see points at 10 s) than after dark adaptation (points at > 10 min). The rapid decay of the 515 nm change slowed as the leaves adapted to darkness, and the change from rapid to slow decay had a half-time of about 5 min. These effects were similar to those seen by Morita et al. [20,21], and Vallejos et al. [22]. However, in contrast to the report by Morita et al.

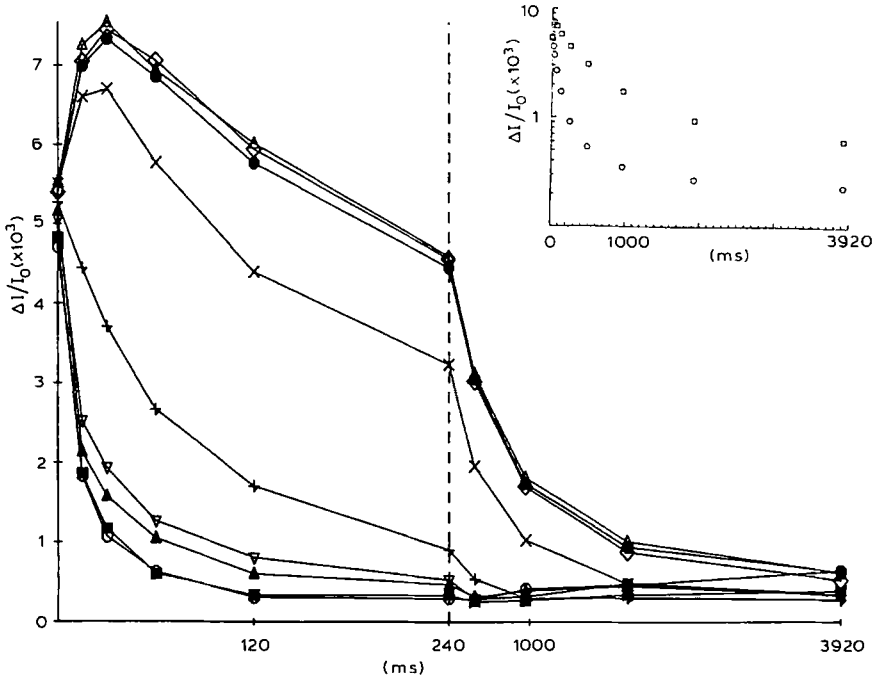

Fig. 1. Effects of light and dark adaptation on the decay kinetics of the flash-induced electrochromic shift. An intact cucumber a leaf was dark-adapted for 2 h then light-adapted with 400 half-saturating actinic xenon flashes (20 Hz). At various times after this actinic flash train, a single actinic flash was given and the electrochromic shift was measured using the portable double-flash kinetic spectrophotometer described in Ref. 24: ○, 10 s; ■, 30 s; ▲, 70 s; ▽, 150 s; +, 310 s; ×, 630 s; ●, 1270 s; ◇, 2550 s; △, 5110 s after the actinic flash train. Note the split in the time base to allow resolution of both fast and slow kinetics on the same figure. Inset: Semilogarithmic plot of the decay kinetics of the flash-induced electrochromic shift in a dark-adapted leaf (□), or in a light-adapted leaf (400 actinic flashed at 20 Hz, then 2 min dark time to allow dissipation of the pmf) (○) after a single flash.

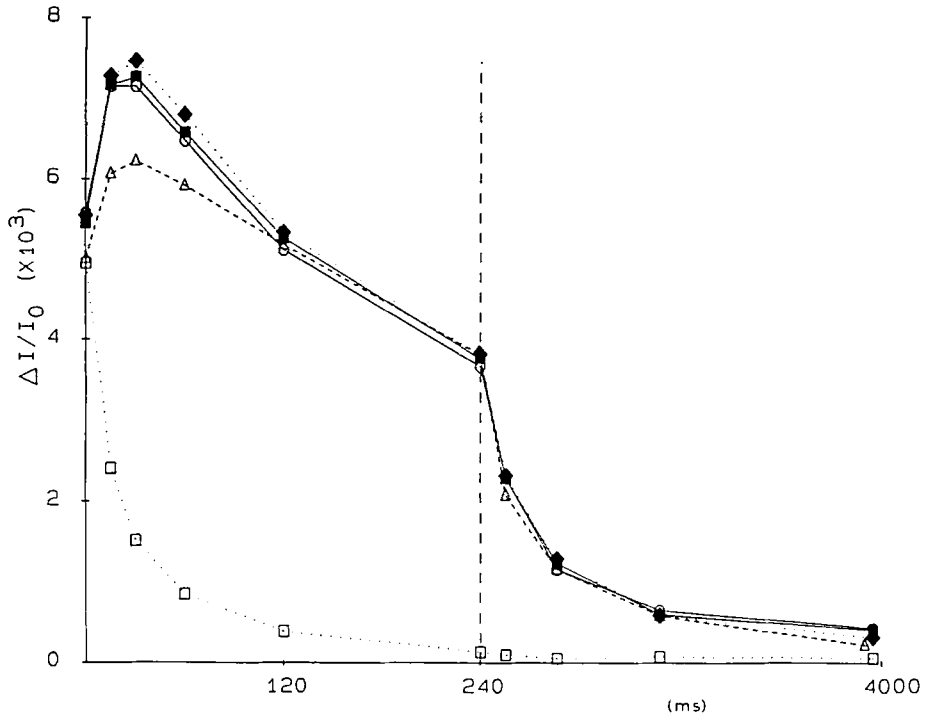

Fig. 2. The effects of methyl viologen and DCCD on the decay rate of the electrochromic shift. Previously dark-adapted leaves (■) and those light-adapted by 400 actinic flashes (□), followed by 2 min dark time to dissipate the pmf, light-adapted leaf treated with methyl viologen (○), dark-adapted leaf treated with methyl viologen (◆) and light-adapted leaf treated with DCCD (△). The methyl viologen-treated leaves were lightly abraded with 400 grit Carborundum then were applied with a tissue soaked in 0.1% Tween 80 and 3 mM methyl viologen. The control leaf was similarly treated except that methyl viologen was not included.

[20], we were unable adequately to fit the decay of the electrochromic shift of either light- or dark-adapted leaves to one or two exponential curves (see Fig. 1, inset). This probably reflects the ability of the instrument used in the present work to measure changes with a high sensitivity and a minimal actinic effect of the measuring beam over an extended time. For this reason, we chose the amplitude of electrochromic shift 240 ms after the actinic flash as a relative indicator of the extent of the slowly decaying phase (referred to hereafter as the 'slow phase'). At this time, the rapidly decaying phase (the 'rapid phase') of the decay appears complete, and a large fraction of the slow phase remains to account for the rest of the decay.

Effects of DCCD and methyl viologen on the 515 nm decay kinetics

In order to confirm the causal connection between the activation of the ATPase, and the decay of the 515 nm change, we have investigated the effects of infusing DCCD or methyl viologen into the leaves, prior to assay.

In Fig. 2, the effects of DCCD and methyl viologen on the decay kinetics of leaves in the light- or dark-adapted state are shown. After infusion with DCCD, the decay of the 515 nm electrochromic change following a single flash was slow, and was not affected by pre-illumination. However, the amplitude of the fast (rising) phase, and both the amplitude and rise kinetics of the slow (rising) phase in the dark-adapted case, were essentially unmodified by DCCD, showing that the inhibitor had no appreciable effect on the photochemical or dark electron-transfer reactions through which the pmf was generated. In view of the well-characterized effect of DCCD in inhibiting the ATPase, the results are consistent with the suggestion that the major flux by which the pmf is dissipated is that of protons through the ATPase. If this is the case, the difference in the kinetics with and without DCCD can be used to estimate the flux through the ATP-ase, and by extrapolation, the rate of ATP synthesis in the intact leaf.

The transition from the dark- to the light-adapted kinetics of the electrochromic shift is most probably due to the greater flux of protons through the ATPase on activation by reduction of the γ-subunit SH groups through the TR system. To test this hypothesis, the effect on this transition of treating the leaf for 30 min with 3 mM methyl viologen was measured, as shown in Fig. 2. The methyl viologen is a low-potential electron acceptor that competes with ferredoxin for electrons from PS I and shuttles them to atmospheric oxygen. This herbicide therefore prevents the reduction of TR, and thus acts as an inhibitor of enzymes activated through reduction by TR [16]. Treatment with methyl viologen resulted in complete loss of the effects of varying preillumination conditions on the decay of the

515 nm change, as would be expected from the above. It has been previously observed that treatment of leaves with methyl viologen also prevents preillumination-induced activity in the chloroplast ATPase [26,27] and other chloroplast enzymes [28].

Time-course of deactivation of the ATPase

The time-course of deactivation of the ATPase, as indicated by the increase in the extent of the slow phase of decay is sigmoidal (Fig. 3). With increasing periods of light adaptation, the onset of the sigmoidal rise was delayed, to form a family of sigmoidal curves. After several seconds of exposure to room light, the kinetics seen in these experiments resembled the sigmoidal kinetics for the dark deactivation of ATPase activity and the disappearance of the rapid decay phase of electrochromic shift found by Morita et al. [21]. On illumination with brighter light for periods longer than a few seconds, the kinetics of the sigmoidal phase were progressively distorted (not shown).

The points in Fig. 3 taken shortly after the flash show a small, relatively fast increase in the amplitude of the slowly decaying phase which was completely by about 60 s. This was probably not due to changes in the redox state of the ATPase, but to the fact that the pmf induced by the pre-illuminating flashes had not fully decayed by this time. The baseline for each trace from which the points in Fig. 3 were derived was taken immediately before the single actinic flash used to assay the decay kinetics of the 515 nm change, and this baseline was subtracted from all points in the trace. The presence of a membrane potential before the assaying flash (giving rise to a pre-existing electrochromic shift) would be expected to cause such an offset in the curve, since this pre-existing pmf would have decayed during the assay period. From this result, it seems likely that the effect of any pmf induced by preillumination must have decayed within about 1 min, but that the effects of the preillumination in inducing reduction of the ATPase followed a much slower time-course, especially after larger numbers of actinic flashes.

As mentioned above, the length of the lag before the sigmoidal rise increased with increasing numbers of actinic flashes, and was longest after extended exposure to room light. Since the lag is associated with the continued presence of activated ATPase, we may assume that the γ-subunit sulfhydryl groups remained in the reduced state during this time. The simplest explanation for this behavior is that, in the intact leaf, the redox state of the γ-subunit sulfhydryls reached equilibrium with a large buffering redox pool with a somewhat lower midpoint potential. Reduction of a relatively small fraction of this pool by a relatively small number of actinic flashes would reduce a large fraction of the γ-subunit sulfhydryls. Thus, as shown in Fig. 3, after only 80–100 actinic flashes, the ATPase reached a maximal level of activation, and could not be further reduced. During dark-adaptation, the buffering redox pool would become oxidized by oxygen or by turnover of oxidizing pathways. However, a large fraction of the pool would have to be oxidized before a significant fraction of the γ-subunit sulfhydryls would begin to be oxidized, causing a time lag between oxidation of the redox pool and the decrease in activity of the ATPase. This time lag would be expected to increase with increasing numbers of actinic flashes, since a larger frac-

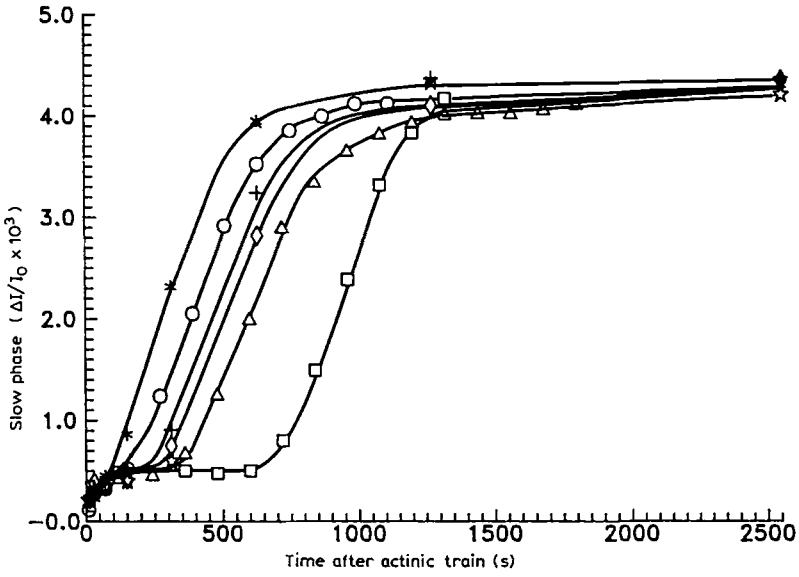

Fig. 3. The kinetics of recovery of the slow decay phase of the electrochromic shift during dark-adaptation. A leaf was dark-adapted for 2 h then illuminated by various numbers of actinic flashes at 20 Hz or by exposure to room light for several seconds. The slow phase was measured as the amplitude of electrochromic shift 240.2 ms after a single actinic flash given at various times after the initial actinic flash train; *, 100 actinic flashes; ○, 200 actinic flashes; +, 400 actinic flashes; ◇, 800 actinic flashes; △ 1600 actinic flashes; □, several seconds exposure to room light.

tion of the buffering pool would have to be reoxidized before the γ-subunit ulfhydryls could be oxidized, and this effect is readily seen in Fig. 3.

By observing the decay of the electrochromic shift in intact leaves, and the ATPase activity in chloroplasts freshly isolated from intact leaves at different times during dark-adaptation. Morita et al. [21] found that deactivation of the ATPase upon dark adaptation followed a sigmoidal curve with a half-time of about 25 min. In isolated chloroplasts, however, the deactivation of the ATPase activity followed a single exponential curve with a half-time of about 8 min. This group also

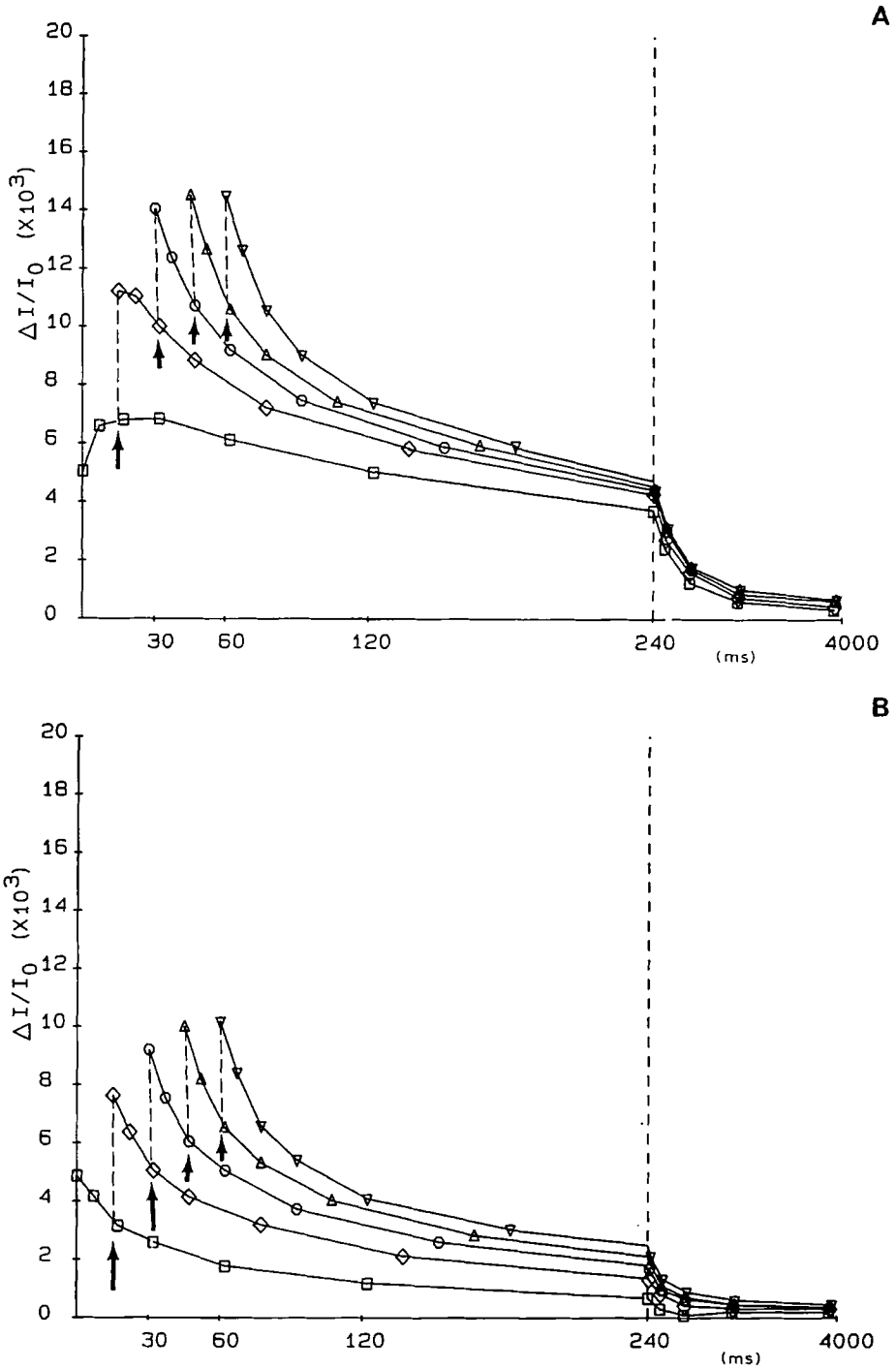

Fig. 4. Kinetics of electrochromic shift after multiple closely spaced actinic flashes. Multiple actinic flashes were given to either dark-adapted control leaves (4a), control leaves that were light-adapted by 400 actinic flashes at 20 Hz, followed by 2 min dark time to dissipate the pmf (4b), or leaves treated with 0.5% dicyclohexylcarbodiimide (DCCD) in 0.5% Tween 80 then dark-adapted for 2 h (4c). The actinic flashes were given 15 ms apart and the kinetics of the electrochromic shift were measured at 515 nm. The first actinic flash was given at the 0 time point and subsequent flashes were given at the arrows. Note the split time base to allow resolution of both the rapid and slow kinetics on the same figure.

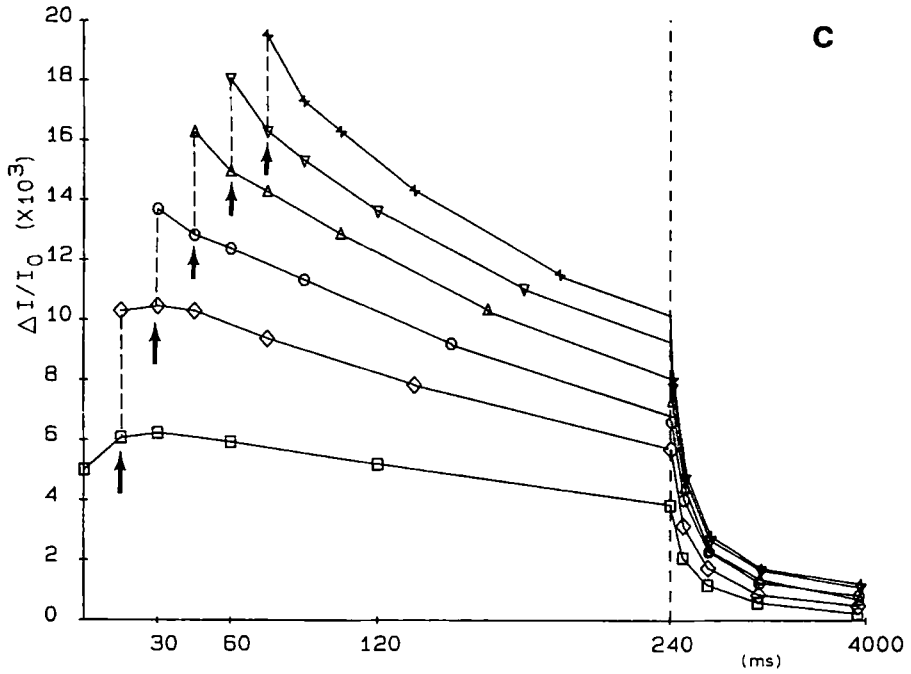

Fig. 4 (continued).

attributed the increase in amplitude of the 'slow phase' to the re-oxidation of the γ-subunit sulfhydryl groups of the ATPase. They suggested that dark deactivation and light activation are suppressed by factors or conditions that were lost during isolation. We suggest that the factor lost in the isolated chloroplast is a large redox buffering pool. Since ATP does not quickly penetrate the chloroplast envelope (reviewed in Ref. 29), it is most probable that in the studies of Morita et al. [21], the ATPase activity measured in isolated chloroplasts (initiated by exogenous ATP addition) was predominantly from broken chloroplasts which had lost their soluble stromal contents, including NADPH, TR, and FD. It should be noted that, in contrast to the results with spinach, Mills and Mitchell [27] found that isolated pea thylakoids exhibited a much slower reoxidation of the ATPase compared to that of intact chloroplasts. It seems probable that, at least in peas, a factor responsible for the oxidation of the γ-subunit sulfhydryls is lost during isolation.

A deviation from the simple two-pool model described above occurs when plants are taken directly from the growth chamber (600–800 $\mu E \cdot m^{-2} \cdot s^{-1}$) or when dark-adapted plants are light-adapted for more than 15 min under saturating illumination. In these cases, the recovery of the slow phase follows longer, more complex kinetics (data not shown). We surmise that under extended illumination with high intensity light, the enzymes involved in the Calvin cycle will become activated and the levels of intermediates of this pathway will change. The redox state of the large metabolite pools might come into equilibrium with that of the γ-subunit of the ATPase. The altered kinetics of recovery of the slow phase under these conditions could possibly reflect the deactivation of these enzymes, and/or the changing levels of intermediates of the Calvin cycle which are expected to be different under bright illumination.

Effects of varying pmf in light- and dark-adapted leaves

In order to follow the effect of varying pmf under conditions of light and dark adaptation, it was necessary to chose 'standard' conditions. For standard 'dark-adapted' leaves, we chose 2 h of dark adaptation, since further dark adaptation had no effect on the kinetics of the decay of the electrochromic shift. On the basis of the data in Fig. 3, we chose leaves treated with 400 actinic flashes (at 20 Hz), followed by a 120 s dark adaptation as 'light-adapted' leaves. After 400 actinic flashes, the lag time before onset of oxidation of the γ-subunit of the ATPase was sufficiently long that leaves could be left for 120 s to allow complete dissipation of the preillumination-induced pmf without any significant appearance of the slow decay phase associated with the oxidized ATPase.

It was also necessary to establish that a number of flashes sufficient to generate a maximal pmf did not lead to reduction of the ATPase. In the dark-adapted leaf, 1–6 of the 50% saturating actinic flashes did not appear to cause significant reduction of the γ-subunit sulfhydryls, since, after up to 10 actinic flashes, followed by 2 min of darkness to dissipate the pmf, the

decay of the single actinic flash-induced electrochromic shift was, within experimental noise, indistinguishable from that of a completely dark-adapted leaf (data not shown).

In the experiments shown in Fig. 4a–c, different numbers of closely spaced actinic flashes (15 ms apart) were given to light- and dark-adapted leaves in order to observe the relationship between the amplitude of the electrochromic shift and its decay rate in leaves with reduced and oxidized γ-subunit sulfhydryls. The traces for the light- and dark-adapted leaf form two families of curves. In the dark-adapted leaf (Fig. 4a), a rapid decay phase was seen only with two or more closely spaced actinic flashes, whereas in the light-adapted leaf, a single actinic flash was sufficient to induce a rapid phase of decay (Fig. 4b). If we argue that the rapid decay is due to flux through the ATPase, we can interpret these data as showing different threshold levels for activation of the ATPase in the light- and dark-adapted leaves, as suggested by Junesch and Gräber [13]. When either light- or dark-adapted leaves were treated with 0.5% DCCD the rapid decay was eliminated, indicating that under both conditions, the increase in the decay rate above the threshold amplitudes was probably due to flux through activated ATPase (Fig. 4c; the kinetics for the light-adapted leaf were essentially the same as those for the dark-adapted leaf shown).

In isolated thylakoids [13] and in an isolated, reconstituted system [30], reduced ATPase show a lower activation threshold than the oxidized ATPase. This is reflected in a non-ohmic relationship between the driving force across the membrane (pmf, either as a ΔpH or as a $\Delta\psi$) and the rate of ATP synthesis (cf. Fig. 3 of Ref. 30). This is also reflected in plots of the number of actinic flashes given to thylakoids vs. the amount of ATP synthesized, the amount of tightly bound ADP released and the ATPase activity [31]. These types of plots demonstrate that, because of the regulation of the ATPase, an energetic threshold above that of ΔG_{ATP} must be overcome before the ATPase becomes active in ATP synthesis. Below this threshold, the rate of ATP synthesis is kinetically limited [18,32,33].

If the stoichiometry of H^+/ATP flowing through the ATPase is constant, the rate of decay of the electrochromic shift should also indicate the rate of ATP formation, since the movement of a proton through the ATPase represents a current across the membrane. By this argument, a plot of the amplitude of the electrochromic shift against the decay rate at that amplitude should follow a relationship similar to that of plots of pH vs. the rate of ATP synthesis. Fig. 5 is an example of such a plot derived from the data in Figs. 4a–c. This plot follows, at least qualitatively, the same non-ohmic behavior as seen in plots of ΔpH vs. the rate of ATP synthesis. Apart from a few points on the graph derived from data measured close to the actinic flash, which show deviations from the rest of the curves due to the slow rise of the electrochromic shift that occurs in that time scale, the data from experiments with different numbers of actinic flashes fall on three well-defined curves. It is striking that the shape of the curve does not appear to depend significantly on the number of actinic flashes, but only on the condition of the ATPase, depending on pre-illumination (and hence state of activation) or inhibition by DCCD. This means that we can

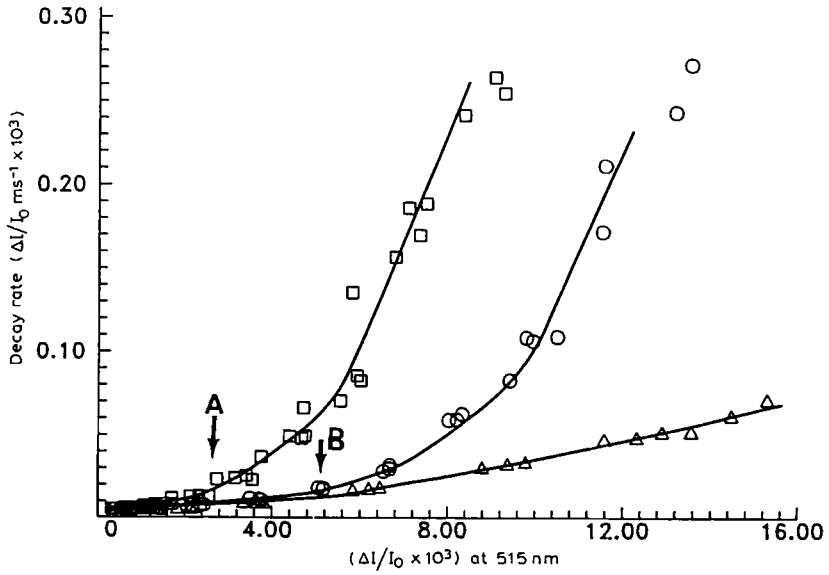

Fig. 5. Dependence of decay rate of the electrochromic shift on its amplitude. This data was derived from that in Fig. 4a–c as follows. The amplitude plotted on the *X*-axis represents the average of the amplitudes of two successive time points for a kinetic curve and the rate of decay is the slope of the line between these two successive points. The data in the curves are taken from the kinetic curves in Fig. 4 with the exception of negative slopes due to the slow rise of the electrochromic shift; ○, dark-adapted leaf; □, light-adapted leaf; △, leaf treated with DCCD.

effectively separate the effects of preillumination and pmf.

The threshold level of pmf required for activation of the ATPase, as indicated by the amplitude of the electrochromic shift at which an increase in decay rate over the DCCD level was apparent, was much lower for the light-adapted (Fig. 5, arrow A and $\Delta I/I_0 = 3 \cdot 10^{-3}$) than for the dark-adapted leaf (Fig. 5, arrow B at $\Delta I/I_0 = 5 \cdot 10^{-3}$). Furthermore, as in the plots of ΔpH vs. ATP synthesis, above the threshold levels, the relationships between the amplitude of electrochromic shift and its rate of decay are similar. When either light- or dark adapted leaves are treated with 0.5% DCCD, most of the non-ohmic behavior seen in the native leaves is eliminated, indicating that the majority of the non-ohmic response is due to flux through the ATPase.

We attempted to distinguish the flux of protons through the ATPase from that through other pathways by subtracting the flux due to passive decay (that decay insensitive to DCCD). If the only effect of DCCD were to block the flow through the ATPase, then the difference in these fluxes should yield the DCCD-sensitive flux. From each pair of data points in a kinetic curve, we obtained a slope. Since the decay rate of the electrochromic shift in the presence of DCCD is dependent upon its amplitude, we subtracted from these slopes, the slope of the DCCD-treated leaf at a corresponding amplitude interpolated from the data presented in Fig. 5. Using these 'corrected' slopes, we reconstructed the kinetic curves. Some examples of these reconstructed curves are shown in the inset to Fig. 6. We feel the procedure worked fairly well, since the electrochromic shift of both light- and dark-adapted leaves decayed rapidly to certain non-zero levels (that of light-adapted leaves decayed to a much lower level than that of dark-adapted leaves) after which no significant decay can be observed.

We then estimated the total amount of charge separation induced across the thylakoid membrane by different numbers of actinic flashes. The slow rise in the amplitude of the electrochromic shift (phase 2) was assumed to be proportional to that of the fast rising phase (phase 1). Since after one actinic flash in a dark-adapted leaf, there was no significant flux through the ATPase (see Fig. 6, inset), the relative amplitudes of phase 1 and 2 (phase 1 : phase 2 $\approx$ 2 : 1) were estimated from these data. The flash number dependence of the difference between the estimated total charge separation and the amount of this estimated to have decayed by ATPase turnover for light- and dark-adapted leaves is shown in Fig. 6. Extrapolation to zero energy flux through the ATPase shows that the reduced enzyme would become fully activated by the amount of light in a fraction of a half-saturating actinic flash while more than one of these actinic flashes is required to activate the oxidized enzyme. Furthermore, the slopes of the two

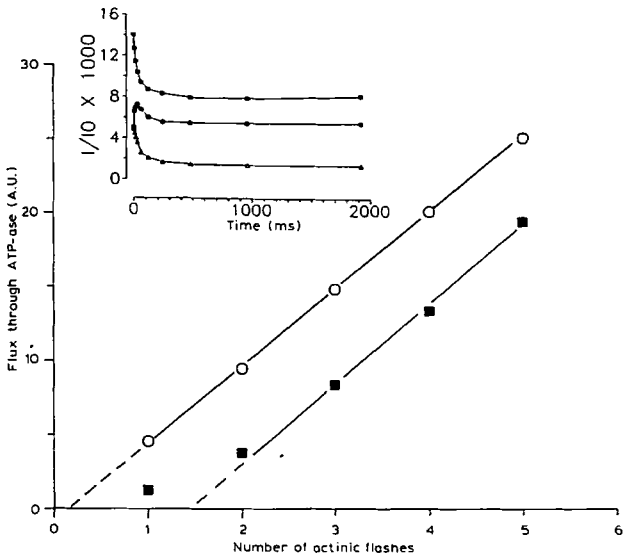

Fig. 6. Actinic flash number dependence of the energy flux through the ATPase. The flux of energy through the ATPase was derived from the data in Figs. 4 and 5 as described in the text, ○, light-adapted leaf; ■, dark-adapted leaf. Inset: Example traces of kinetics traces from Fig. 4a and b, corrected for the slowly decaying phase as described in the Discussion section; ●, dark-adapted leaf after one actinic flash; ■, dark-adapted leaf after two actinic flashes spaced 15 ms apart; △, light-adapted leaf after one actinic flash.

lines, above the number of flashes required to activate the enzymes are the same in the dark- and light-adapted leaves, indicating that the efficiency of ATP-synthesis above the activation threshold in the dark- and light-adapted leaves is the same. This is in good agreement with the interpretation derived from the rate of decay of the electrochromic shift.

Discussion

The results in this paper are consistent with the model previously proposed by Junesch and Gräber [13]. The scheme in Fig. 7 shows how each of the four states proposed in this model has been characterized in the present work by measurement of the decay kinetics of the flash-induced electrochromic shift.

Our results show that the ATPase in the dark-adapted leaf is mostly in the state E_i^0 (i.e., the subunit sulfhydryls are oxidized and the ATPase is not activated by any existing pmf). In this state, activation can be achieved by 2–3 half-saturating actinic flash, giving rise to an electrochromic shift of amplitude $\Delta I/I_0 = (4\text{–}5) \cdot 10^{-3}$, which indicates the pmf required to activate the ATPase to the state E_a^0. After activation, the onset of a rapid decay (Fig. 4a) and DCCD-sensitive flux (Fig. 6) are observed. This amplitude corresponds to the relatively high threshold pmf required for activation of the oxidized ATPase in the model of Junesch and Gräber [13].

Preillumination with relatively weak light (a few seconds of room light, or a larger number of actinic flashes (80 or over at 20 Hz)) caused both the reduction of the γ-subunit sulfhydryls and the activation of the ATPase by pmf. This would have converted the ATPase to the state E_a^r in the scheme. Upon dissipation of the preillumination-induced pmf by dark-adaptation for 2 min, the state E_a^r decays to the reduced, inactive state, E_i^r. This state could be converted to the active E_a^r by only one actinic flash as shown by the decay rate of the electrochromic shift (Fig. 4b), or the DCCD-sensitive flux (Fig. 6). In this state, the threshold for onset of the rapid decay of the electrochromic shift was indicated by an electrochromic change of amplitude $\Delta I/I_0 \approx 3 \cdot 10^{-3}$.

Models for the kinetic behavior leading to oxidation of the ATPase

In the dark, the state E_i^r decayed to E_i^0 with a half-time which had a minimal value of about 200–300 s when activation was achieved with minimal preillumination, but was preceded by a lag which was dependent on the amount of preillumination. The simplest hypothesis to explain this dependence is that the γ-subunit sulfhydryls represent a relatively small redox pool which equilibrates with a large redox buffering pool of somewhat lower midpoint potential, possibly the $NADP^+$/NADPH couple or the glutathione couple. The regulatory thiol groups of some chloroplast enzyme thioredoxins may equilibrate with the $NADP^+$/NADPH couple through FD [16] and with thiol couples such as glutathione directly [34]. Depending upon assumptions of the kinetics of oxidation of the pools, our evidence suggests that this redox buffering pool should have a midpoint potential between about 14 and 20 mV lower than that of the sulfhydryls of the γ-subunit, as discussed below.

Reasonable candidates for the redox buffering pool can be suggested. These should include the $NADP^+$/NADPH couple which has an $E_{m,7} = -0.32$ V and the glutathione couple which has an $E_{m,7} = -0.34$ V. The γ-subunit sulfhydryls are expected to have a value for $E_{m,7}$ somewhere in the range found for thioredoxins, which have been found to vary between −0.24 V for that of yeast [35] and about −0.3 V for thioredoxin m from *Zea mays* [36]. The γ-subunit sulfhydryls are reduced by DTT [16], which has an $E_{m,7} = -0.33$ V [37], and apparently by lipoic acid, since addition of excess completely activated the ATPase [38]. Since lipoic acid has an $E_{m,7} = -0.27$ V [37], we expect the $E_{m,7}$ of the γ-subunit sulfhydryls to be similar to or higher than this. Even with the light-induced rise in the stromal pH from 7 to 8 [39], which would lower the potential of the γ-subunit sulfhydryls relative to the $NADP^+$/NADPH couple by 0.03 V per pH unit, the difference in midpoint potentials between the $NADP^+$/NADPH couple and the γ-subunit sulfhydryls would be at least 0.02 V in the light and 0.05 V in the dark. These are both significant differences since the reduction of the sulfhydryls and of $NADP^+$ are both two-electron processes, showing a 10-fold change in oxidized to reduced ratio over a 30 mV range. Models to account for the kinetic and redox behavior of the system are discussed below.

Since we have at present no independent measure of the kinetics of the redox pools involved, we can only state some of the more likely interpretations and some general consequences of these. There are at least two simple cases that cannot easily be distinguished with our present data: the case where the pools are oxidized linearly with time and the case where they are reoxidized with a pseudo-first order rate constant. The former case would be expected if the electrons were lost through a rate-determining step from the smaller, less reducing pool. The later case would be expected if the electrons were lost from the larger, more reducing pool.

In the case where the pools are reoxidized linearly with time, the extent of reduction of the buffering pool can be estimated from the lag before onset of oxidation of the γ-subunit of the ATPase (as indicated by the onset of the recovery of the 'slow phase', the sigmoidal kinetics in Fig. 3). Assuming that the degree of reduction of the pool is proportional to the number of actinic flashes in the pre-illuminating flash train, a plot of the number of actinic flashes against the lag time before onset of the recovery of the slow phase should be linear until the larger pool is significantly reduced, where saturation effects should become apparent. Our data can be fit fairly well to a straight line for about 300 actinic flashes, above which the points deviate significantly from linearity, indicating (in this model) that the large redox buffering pool is becoming filled above 300 actinic flashes. Exposure to room light for 20 s or more gives the largest lag time. It is therefore reasonable to assume that several minutes exposure to room light should completely fill the larger pool. The ratio between the lag time, and the time from the onset of recovery of the slow phase to its completion, should therefore represent the ratio of the size of the large buffering pool to that of the pool associated with the γ-subunit of the ATP-ase. This pool may include not only the γ-subunit itself, but any other redox carrier in equilibrium with the γ-subunit and with a similar midpoint potential and redox chemistry. For example, if TR has a similar midpoint potential, it may also be included in this pool. From this model the ratio of the large pool size to that of the pool associated with the γ-subunit of the ATPase is about 3 : 1. A half time for the reoxidation of the large pool can be estimated at about 10 min. We also find that at the point where the slow phase is half-reoxidized, the larger pool is about 80% oxidized. Assuming a two-electron redox chemistry for the two pools, this gives an estimated difference in midpoint potentials of about 14 mV.

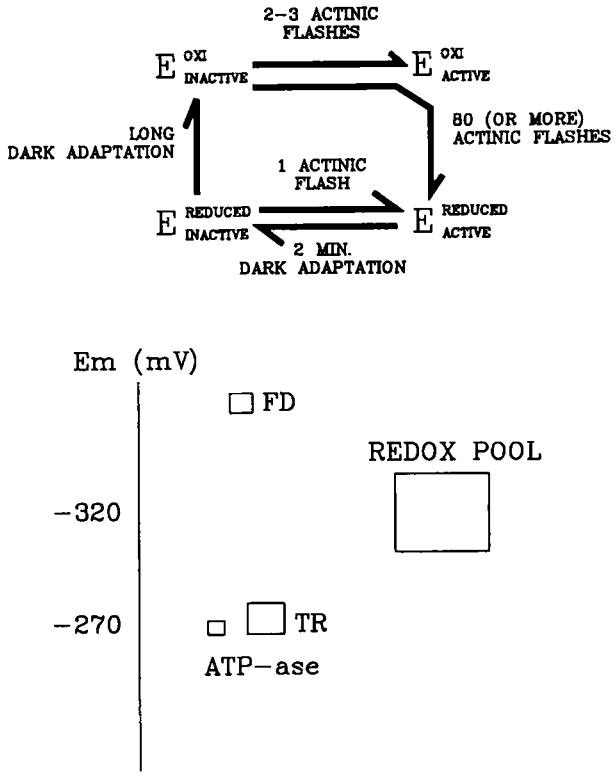

Fig. 7. Scheme for ATP-ase activation based on the kinetics of the 515 nm change. This scheme is adapted from that of Gräber and co-workers. The ATPase, E, can be in four states: two inactive and two active. A disulfide bond on the enzyme complex can be either oxidized or reduced and both of these forms are inactive until an electrochemical gradient, pmf, is imposed across the thylakoid membrane. The maximal rates of ATP synthesis (or decay of electrochromic change) are the same for both active forms, but, the magnitude of pmf required to activate the two forms differs, the oxdized form requiring a greater pmf for activation than the reduced form. This is reflected in the electrochromic change as follows. A completely dark-adapted leaf, presumably with the oxidized, inactive ATP synthase, E_i^0, requires a greater electrical potential, (i.e.: more actinic flashes) to induce a rapid decay of the electrochromic shift by activating E_i^0 and E_a^0 (Fig. 4a and 5). A large number of actinic flashes both reduces and activates the coupling factor to the state E_a^r. A short (2 min) dark-adaptation is sufficient to collapse the electrochemical gradient but is not sufficient to oxidize the coupling factor. Therefore, the enzyme reaches the state E_i^r. In this state, very little light – only one sub-saturating actinic flash – is required to activate the coupling factor, resulting in a rapid decay of the electrochromic change (Figs. 3b and 4). Below this scheme is a model representing the approximate redox potentials of the components involved in the regulation of the ATPase; FD, ferredoxin; TR, thioredoxin. The sizes of the boxes represent the relative sizes of the redox pools they represent. The derivation of this model is described in the text.

It is also possible to make these estimates using the alternative model, in which reoxidation of the larger pool follows pseudo-first order kinetics. The time, t, required to oxidize the pool from one fraction reduced, f_1, to another, f_2, is:

$$t = -\frac{1}{k}\ln\frac{f_1}{f_2}$$

where k if the pseudo-first-order rate constant. The point at which the γ-subunit of the ATP-ase would be oxidized in 50% of centers, as reflected in the point at which the slow phase has recovered by 50% in Fig. 3, should reflect the point at which the redox pool reaches some particular, consistent value. The time required to reach this point should be dependent upon the amount by which the pool was reduced at the start of the decay. Assuming that the degree of reduction of the pool is proportional to the number of actinic flashes in the preilluminating flash train, a plot of the log of the number of actinic flashes in the actinic train vs. the time from the end of the actinic flash train to the point where the slow phase has recovered by 50%, should give a straight line, the slope of which should indicate the decay rate constant. We find a fairly good fit to a single exponential with a rate constant of 0.01 s^{-1} (not shown).

From the curves in Fig. 3, the time taken for the γ-subunit pool to change from 25% oxidized to 75% oxidized – a change in the percent oxidation of 3-fold – is found to be about 250 s. Using the value for k of 0.01 s^{-1}, estimated above, we find that, in the same amount of time, the larger pool is oxidized by about 92%. Given that these two pools are in equilibrium and assuming a 2-electron redox chemistry for both pools, we estimate that the E_m of the redox buffering pool is about 20 mV more negative than that of the γ-subunit sulfhydryls.

A lag time before recovery of the slow phase is only seen after pre-illumination with more than about 80 actinic flashes (50% saturating) at 20 Hz (see Fig. 3). Therefore, 80 actinic flashes can be thought of as essentially completely reducing the redox pool associated with the γ-subunit of the ATPase. Over the range from 100 to 1600 actinic flashes, we find a fairly linear relationship when the log of the number of actinic flashes is plotted as a function of the time for recovery of the slow phase by 50% (not shown). At the point where the electrons delivered by linear electron flow significantly reduce the large redox buffering pool, this relationship would be expected to deviate from a straight line. It is possible that, at larger numbers of actinic flashes, turnover of oxidizing pathways might prevent the saturation of the pool. By this rough estimate, the ratio of 1600 : 80 (20 : 1) should give the lower limit to size of the redox buffering pool relative to that of the pool associated with the γ-subunit of the ATPase.

These two interpretations give different estimates of the relative pool sizes, half-time of reoxidation and relative midpoint potentials. In addition, other interpretations may be possible, including other regulatory mechanisms (e.g., a regulatory protein, as in mitochondria [40] or the binding of nucleotides [41]) not included in the model of Junesch and Gräber [13]. Measurements of the reoxidation rates of the various redox pools in the dark would be very helpful in better establishing the mechanism of the reoxidation of the

γ-subunit. With this information, measurements similar to those in Fig. 3 may provide a useful probe of the redox state of important regulatory intermediates in the intact plant.

Both these models are in contrast to the hypothesis of Quick and Mills [33] who suggested that, although the redox state of the γ-subunit sulfhydryls was in equilibrium with the small TR pool, the rate of re-oxidation of the sulfhydryls was kinetically limited by the rate of TR reoxidation. This hypothesis cannot easily explain the dependence of the lag time on the amount of preillumination (Fig. 3). Quick and Mills [33] adduce as support for their hypothesis the relatively 'rapid' oxidation of the $NADP^+/NADPH$ pool by turnover of the Calvin-cycle. However, in view of the relatively rapid forward rates, and values for equilibrium constants in the range 10–100, it seems more probable that the redox state of the γ-subunit sulfhydryls would reach equilibrium with the $NADP^+/NADPH$ pool through FD within the very long time frame of the dark-adaptation. Furthermore, by having the midpoint potential of the γ-subunit sulfhydryls somewhat higher than that of the $NADP^+/NADPH$ pool, only a small fraction of the pool would have to remain in the reduced state to maintain a significant level of reduction of the γ-subunit sulfhydryls. There have been few studies on the redox state of the $NADP^+/NADPH$ pool upon darkening in intact systems. However, one report by Lendzian and Bassham [42] suggests that about 10% of the pool may remain reduced 5 min after illumination, the longest time point measured. This question could be answered by simultaneous measurements of the redox state of the $NADP^+/NADPH$ pool and the activation threshold of the ATPase.

Estimation of the $\Delta\psi$ required to activate the oxidized and reduced ATPase

In isolated intact spinach chloroplasts, Zickler et al. [43] found that one saturating single-turnover actinic flash produced a potential difference of about 100 mV across the thylakoid membrane. The extent and kinetics of the electrochromic shift induced by the approximately half-saturating actinic flashes used in this study is almost identical for cucumber and spinach leaves (data not shown). We therefore began with the assumption that about 50 mV is produced by a single half-saturating actinic flash in the present system, and used this to calibrate the electrochromic shift.

In order to simplify the estimation of the pmf, we assumed that the pH buffering of the lumen is high, and the capacitance of the membrane is comparatively low, so that the pmf generated by a few actinic flashes consists mostly of the $\Delta\psi$ component. Evidence for this comes from measurements of intrathylakoid pH changes following saturating single-turnover flash excitation using neutral red [44], or electron spin resonance probes [45]. These studies found a pH change of approx. −0.06 units per saturating single-turnover actinic flash, and that the buffering capacity of the lumen was fairly constant. This can be compared to the approx. 100 mV of electrical potential (energetically equivalent to about 1.7 pH units) generated by similar flashes, based on the estimates of Zickler et al. [43]. Furthermore, Hangarter et al. [31] found that between 12 and 13 saturating actinic flashes were required to activate the reduced ATPase of isolated spinach thylakoids treated with nonactin to collapse the $\Delta\psi$ component of the pmf, whereas in the present work, in intact leaves with the $\Delta\psi$ component of the pmf unhindered, we found that the reduced ATPase is activated with only one half-saturating actinic flash.

With these assumptions, we estimate that the reduced ATPase becomes activated with a $\Delta\psi$ of about 50 mV (equivalent to about 0.8 pH units if the pmf were composed solely of a pH) and the oxidized ATPase becomes activated at about 100 mV (equivalent to about 1.7 pH units). This suggests that the reduction of the ATPase lowers the energetic threshold for activation by about 50 mV (equivalent to about 0.8 pH units). The value of about 1 pH unit for the activation of the reduced ATPase is paradoxical because this amount of pmf would drive a ΔG_{ATP} of only about 17 kJ/mol ATP, using a stoichiometry of 3 H^+/ATP (for example, see Ref. 46 where $n = 2.84$). The ΔG_{ATP} measured in chloroplasts ranges from about 42–45 kJ/mol in both dark and illuminated plants [47]. At present we cannot offer any definitive resolution of this paradox. Our results could indicate that one or more of the assumptions made above is inaccurate, or they could suggest that the curves are offset by the presence of a standing pmf, possibly in the form of a ΔpH of about 1 pH unit, across the thylakoid membrane of chloroplasts in the dark in situ as suggested by independent studies [48,49].

A range of values has been cited in the literature for the magnitude by which the energetic threshold for activation of the ATPase is lowered upon reduction of the γ-subunit. Junesch and Gräber [13] found a full pH unit lowering of the energetic threshold upon reduction of the γ-subunit. On the other hand, Ketcham et al. [12] found that the energetic threshold for activation of the ATPase was lowered by only about 0.3 pH units. In this case, an uncounted contribution from $\Delta\psi$ to the pmf cannot be ruled out, since these studies were conducted with continuous illumination without added agents to collapse the electrical potential. Hangarder et al. [31] also found a value of 0.3 pH units, but because of the inherent scatter in the data, a range of values from 0.3 to 0.5 pH units could be obtained (Ort, D., personal communication). Because of the uncertainty of the estimations of the pmf based solely on the $\Delta I/I_0$ at 515 nm in this study, we cannot resolve the apparent discrepancy among the various values cited in the litera-

ture. We are currently conducting further experiments to obtain a better estimate of the magnitude of $\Delta\psi$ required to activate the oxidized and reduced ATPase.

The amount of illumination required for activation and reduction of the ATPase

Finally, one further feature of our experimental results deserves emphasis; the relatively weak illumination required to fully activate the ATPase in situ through the thioredoxin system. Illumination by weak room light for a few seconds, or by a train of 80 flashes at 50% saturating intensity was sufficient to reduce and activate the ATP-ase fully. In view of the long time-scale for deactivation, we may assume that the 40 turns-over of the photosystems induced by the flashes represents a reasonable minimal value. Activation would be achieved by any illumination regime delivering this number of quanta per chain over the 200–300 s of the half-life for deactivation, or a minimal intensity of about 0.2 quanta per center per s. Since the rate-limiting step for photosynthesis is about 10 ms (giving a turnover of 100 s^{-1}), the intensity required for activation is less than 0.2% of the actinic light encountered under normal physiological conditions. It seems unlikely that activation of the ATPase through the thioredoxin system is ever limiting to photosynthesis under normal actinic illumination. Activation by reduction seems to be more of a switch than a regulator and is consistent with the results of Mills and Mitchell [27].

Conclusions

The procedures described here for observing the different states of ATPase may prove to be useful as a means of interpreting these results in terms of an established model [13]. Although these results do not rule out other regulatory mechanisms, we find no evidence to invoke any model more complicated than that presented by Junesch and Gräber [13]. By using the instrumentation developed for this work, we have shown that useful measurement of the ATPase activity under field conditions is straight-forward, and that the method provides a more general probe of the redox and control metabolism on the acceptor side of PS I. Our results suggest that activation of the ATPase through reduction is not normally rate limiting for photosynthesis.

Acknowledgments

We would like to thank Dr. R.R. Wise and Dr. D.R. Ort for providing the plant material for this research. Also, we would like to thank Dr. D.R. Ort and Dr. H. Robinson for many helpful discussions. The research was supported by the U.S. Department of Energy grant DOE-DE-FG02-86ER13594. D.M.K. is currently supported by a McKnight fellowship.

References

1 Mitchell, P. (1961) Nature 191, 144–148.
2 Kaplan, J.H. and Jagendorf, A.T. (1967) J. Biol. Chem. 243, 972–979.
3 Junge, W. (1970) Eur. J. Biochem. 14, 582–592.
4 Smith, D., Stokes, B.O. and Boyer, P.D. (1976) J. Biol. Chem. 251, 4165–4171.
5 Harris, D.A. and Crofts, A.R. (1978) Biochim. Biophys. Acta 502, 87–102.
6 Gräber, P., Schlodder, E. and Witt, H.T. (1979) Biochim. Biophys. Acta 461, 426–440.
7 Strotmann, H., Bickel, S. and Huchzermeyer, B. (1976) FEBS Lett. 61, 194–198.
8 Bickel-Sandkotter, S. and Strotmann, H. (1981) FEBS Lett. 125, 188–192.
9 Ryrie, I.J. and Jagendorf, A.T. (1971) J. Biol. Chem. 246, 3771–3774.
10 Ravizzini, R.A., Andreo, C.S. and Vallejos, R.H. (1980) Biochim. Biophys. Acta 591, 135–141.
11 Nalin, C.M. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7275–7280.
12 Ketcham, S.R., Davenport, J.W., Warnke, K. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7286–7293.
13 Junesch, U. and Gräber, P. (1987) Biochim. Biophys. Acta 893, 275–288.
14 Mills, J. and Mitchell, P. (1984) Biochim. Biophys. Acta 764, 93–104.
15 Wolosiuk and R.A. Buchanan, B.B. (1977) FEBS Lett. 81, 253–258.
16 Buchanan, B.B. (1980) Annu. Rev. Plant Physiol. 31, 341–374.
17 Scheibe, R.A. and Anderson, L.E. (1981) Biochim. Biophys. Acta 636, 58–64.
18 Mills, J. and Mitchell, P. (1984) Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II, pp. 523–526, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht.
19 Witt, H. (1975) Biochim. Biophys. Acta 505, 355–427.
20 Morita, S., Itoh, S. and Nishimura, M. (1981) Plant Cell Physiol. 22(2), 205–214.
21 Morita, S., Itoh, S. and Nishimura, M. (1982) Biochim. Biophys. Acta 679, 125–130.
22 Vallejos, R., Arana, J. and Ravizzini, R. (1983) J. Biol. Chem. 258, 7317–7321.
23 Martin, B. and Ort, D.R. (1985) Photosynth. Res. 6, 121–132.
24 Kramer, D. and Crofts, A. (1988) Photosynth. Res., in press.
25 Joliot, P. and Joliot, A. (1984) Biochim. Biophys. Acta 765, 210–218.
26 Shahak, S. (1982) Plant Physiol. 70, 87–91.
27 Mills, J. and Mitchell, P. (1982) Biochim. Biophys. Acta 679, 75–83.
28 Leegood, R.C., Walker, D.A. and Foyer, C.H. (1985) in Topics in Photosynthesis (Barker, J. and Baker, N.R., eds.), Vol. 6, pp. 189–258, Elsevier/North-Holland, Amsterdam.
29 Heber, U. (1974) Annu. Rev. Plant Physiol. 25, 393–421.
30 Schmidt, G. and Gräber, P. (1987) Z. Naturforsch. 42c, 231–236.
31 Hangarter, R., Grandoni, P. and Ort, D. (1987) J. Biol. Chem. 262, 13513–13519.
32 Schlodder, E., Gräber, P. and Witt, H. (1982) in Topics in Photosynthesis (Barber, J., ed.), Vol. 4, pp. 105–175, Elsevier Biomedical, Amsterdam.
33 Quick, W.P. and Mills, J. (1986) Biochim. Biophys. Acta 851, 166–172.
34 Wolosiuk, R.D. and Buchanan, B.B. (1977) Nature 266, 565–569.
35 Gonzalez, P. Baldesten, A. and Reinhard, P. (1970) J. Biol. Chem. 245, 2363–2370.
36 Rebeille, F. and Hatch, M. (1986) Arch. Biochem. Biophys. 249, 164–170.
37 Clark, W. (1972) Oxidation-Reduction Potentials of Organic Systems, pp. 482–484, Krieger, Huntington, New York.

38 Petrack, B., Craston, A., Sheppy, F. and Farron, F. (1965) J. Biol. Chem. 240, 906–914.
39 Robinson, S. (1985) Biochim. Biophys. Acta 806, 187–194.
40 Pullman, M.E. and Monroy, G.C. (1963) J. Biol. Chem. 238, 3762–3769.
41 Strotman, H. (1986) in Encyclopedia of Plant Physiology (Staehelin, L.A. and Arntzen, C.J., eds.), Vol. 19, pp. 584–594, Springer-Verlag, Berlin.
42 Lendzian, K. and Bassham, J.A. (1977) Biochim. Biophys. Acta 396, 260–275.
43 Zickler, A., Witt, H.T. and Boheim, G. (1976) FEBS Lett. 66, 142–148.
44 Junge, W., Schönknecht, G. and Förster, V. (1986) Biochim. Biophys. Acta 852–93–99.
45 Lavergne, J. (1982) Photobiochem. Photobiophys. 4, 131–144.
46 Junesch, U. and Gräber, P. (1984) Biochim. Biophys. Acta 809, 429–434.
47 Giersch, C., Heber, U., Kobayashi, Y., Inoue, Y., Shibata, K. and Heldt, H.W. (1980) Biochim. Biophys. Acta 590, 59–73.
48 Joliot, P. and Joliot, A. (1980) Plant Physiol. 65, 691–696.
49 Enser, U. and Heber, U. (1980) Biochim. Biophys. Acta 592, 577–591.