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MODULATION OF COUPLING FACTOR ATPase ACTIVITY IN INTACT CHLOROPLASTS

REVERSAL OF THIOL MODULATION IN THE DARK

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Illumination of intact pea chloroplasts results in both the pH activation and the thiol modulation of the reversible protonmotive ATPase, or CF_0-CF_1 (Mills, J.D., Mitchell, P. and Schürmann, P. (1980) FEBS Lett. 112, 173–177). We have studied the reversal of the activation and thiol-modulation processes that occurs when illuminated chloroplasts are darkened. (1) Methyl viologen is shown to prevent light-dependent thiol modulation of CF_0-CF_1 in intact chloroplasts. Studies using methyl viologen indicate that the decline in ATPase activity of intact chloroplasts in the dark is due both to pH deactivation and thiol demodulation of CF_0-CF_1. Reillumination in the presence of methyl viologen reactivates CF_0-CF_1 complexes that have undergone pH deactivation, but does not allow thiol modulation to occur. (2) At 20°C, both pH deactivation and thiol demodulation of CF_0-CF_1 are complete within 10 min darkness. At 3.5°C, both processes are retarded, and require more than 30 min for completion. (3) Lysis of intact chloroplasts greatly retards the thiol demodulation of CF_0-CF_1. In the dark, addition of oxidants to lysed chloroplasts promotes thiol demodulation. Under these conditions, thioredoxin behaves as a weak oxidant, but glutathione has very little effect. Illumination of intact chloroplasts followed by rapid lysis and storage on ice stabilises the pH-activated, thiol-modulated state of CF_0-CF_1 for extended periods in the dark and increases the time required for thiol demodulation to well over 2 h. This method may prove to be useful for preparing thiol-modulated chloroplasts without the need to add or remove exogenous thiols. The results suggest that an oxidative system exists in the stroma which actively reverses thiol modulation of CF_0-CF_1 in the dark. Such a system would be expected if thiol modulation of CF_0-CF_1 were an important physiological process regulating reversible ATPase activity in vivo.

Introduction

It is now established that the activity of several important stromal enzymes can be modulated via changes in the oxido-reduction state of certain of their thiol groups [1–4]. This modulation is widely believed to be implicated in the regulation of photosynthesis in response to a light-dark or dark-light transition [4–6]. Buchanan and co-workers [5,6] have suggested a mechanism for thiol modulation in intact chloroplasts. Upon illumination, electrons from photosynthetic electron transport reduce thioredoxin, a small (mol. wt. approx. 12 000) soluble protein with a redox-active disulphide bridge. Thioredoxin in turn reacts with other soluble enzymes, thereby modulating their activity. Reversal of the thiol-modulation process occurs upon darkening the organelles, and this reversal is thought to be promoted by oxidants such as oxidised glutathione [5], H_2O_2 [7–9] and others as yet unidentified [10].

Abbreviations: CF_0-CF_1, reversible protonmotive ATPase (coupling factor); Tricine, *N*-tris(hydroxymethyl)methylglycine; $\Delta\bar{\mu}_H^+$, difference in the electrochemical potential of protons between the aqueous stromal and intrathylakoid spaces; Chl, chlorophyll; PS, photosystem.

It has been known for many years [11] that the activity of chloroplast coupling factor (CF_0-CF_1) may also be modulated by reduced dithiols, though, in this case, the situation is more complex. The membrane-bound CF_0-CF_1 normally undergoes activation when the stromal or N-side of CF_1 is at about pH 8, and a difference in electrochemical potential of protons, $\Delta\bar{\mu}_{H^+}$, is generated across the coupling membrane [12–16]. We propose to call this type of CF_0-CF_1 activation dual-pH-dependent or simply pH-dependent because, as discussed elsewhere [17], it probably depends on the pH value of the P-pole of CF_1 (that is at the bottom of the proton-well through CF_0) being at a pH of about 5, while the N-side of CF_1 is at the normal pH of the stroma, about pH 8. According to this view, pH activation of CF_0-CF_1 takes place optimally at a dual pH optimum of CF_1 that corresponds to the familiar single pH optimum of soluble enzymes. Conversely, pH deactivation is attributable to the change of protonation state of pH-activated CF_0-CF_1 by displacement of the pH conditions of CF_1 from the dual pH optimum.

Thiol modulation of CF_0-CF_1 is superimposed upon the reversible process of pH activation. Reduced dithiols interact with the pH-activated state of CF_0-CF_1 so that the thiol-modulated enzyme is capable of hydrolysing ATP at high rates [11–13]. In contrast, the non-thiol-modulated, but initially pH-activated CF_0-CF_1 does not hydrolyse ATP at significant rates, though the reasons for this are not yet understood.

Upon illumination of intact chloroplasts (i.e., those that retain undamaged outer envelopes), CF_0-CF_1 becomes both pH activated and thiol modulated [18,19] and ATP hydrolysis is observed in subsequently lysed organelles [19–21]. It has been shown that CF_0-CF_1 may be pH activated and thiol modulated by illuminating broken chloroplasts in the presence of the reconstituted thioredoxin system [20,21], suggesting that this process may account for thiol modulation of coupling factor complexes in vivo.

If thiol modulation of CF_0-CF_1 activity is an important regulatory process in vivo, one would expect it to be reversible, i.e., that thiol demodulation should occur after a light to dark transition. However, studies with broken chloroplasts have indicated that thiol modulation of CF_0-CF_1 activity in the presence of dithiothreitol is virtually irreversible [13,22], or only slowly and partially reversible [16] in the dark.

In this paper we show that CF_0-CF_1 ATPase activity of intact chloroplasts declines in the dark following an illumination. The dark decline of activity is due to two processes: (a) pH deactivation, and (b) thiol demodulation of CF_0-CF_1. Both processes are complete after 10 min in the dark at 20°C, but thiol demodulation is greatly retarded if the organelles are lysed.

Materials and Methods

Intact chloroplasts were isolated from 10–14-day-old pea seedlings (variety Feltham First or Meteor) as previously described [20], except that chloroplasts were washed once in grinding medium. Intactness of the chloroplasts was 60–80% as estimated by ferricyanide permeability of the outer envelope [23].

Experiments were carried out in two stages essentially as described previously [20]. Chloroplasts were initially illuminated (1500 W/m^2 white light) in stirred test tubes containing 0.35 M sorbitol, 30 mM Tricine-KOH, pH 8.0, and 2000 U catalase/ml. Temperature was 20°C. The chloroplasts were either intact, or lysed directly in the activation stage [20] at a concentration of 90–120 μg Chl/ml. When chloroplasts were lysed, the activation stage additionally contained 5 mM $MgCl_2$ in the medium. Aliquots (200 μl) were transferred (using an Oxford sampler) to a hypotonic assay stage where lysis of any intact chloroplasts occurred and ATP hydrolysis was allowed to proceed in the dark.

In most experiments, ATP hydrolysis was assayed by P_i release [20] and the assay stage contained 0.8 ml of 2.5 mM ATP, 2.5 mM $MgCl_2$, 1.25 mM NH_4Cl, 25 mM Tricine-KOH, pH 8.0. In some experiments, ATP-dependent 9-aminoacridine fluorescence quenching was followed, and in this case the assay stage contained 1.2 ml of 2 mM $MgCl_2$, 0.5 mM ATP, 12 μM 9-aminoacridine, 25 mM Tricine-KOH, pH 8.0.

9-Aminoacridine fluorescence was measured on a laboratory constructed fluorimeter. Excitation light at 412 nm was obtained by passing the output of a quartz-iodine lamp though a Hilger-Watts D292 monochromator set at maximum slit width. Fluorescence was detected at right-angles with an EMI 9592 B photomultiplier protected by Wratten 45 and 75 filters and a Corning 4-96 filter.

Thioredoxin$_f$ was a kind gift of Dr. P. Schürmann, University of Neuchâtel [24].

Results

Thiol demodulation of CF_0-CF_1 in intact chloroplasts

The experiments to be described were performed in a two-stage procedure whereby chloroplasts were initially illuminated (activation stage), and small aliquots were taken and assayed for ATPase activity under partially uncoupled conditions in the dark (assay stage, see Materials and Methods for details). We verified earlier reports [13,22,25] that the rates of ATPase activity observed (except for the very lowest [25]) were stable over the time of assay. Thus, the observed rate of hydrolysis is proportional to the fraction of CF_0-CF_1 complexes in their pH-activated, thiol-modulated state at the time of sampling from the activation stage.

Fig. 1 shows how the number of pH-activated, thiol-modulated CF_0-CF_1 complexes varies as chloroplasts in the activation stage are subjected to periods of illumination and darkness. With dark-adapted, lysed chloroplasts to which dithiothreitol has been added, the rate of appearance of ATPase activity is slow on the first illumination, but much more rapid on the second and subsequent illuminations as noted previously by others [13,16,22].

In principle, the kinetics of appearance of ATPase activity can be limited either by the rate at which pH activation is achieved, or by the rate at which reduced thiols modulate the pH-activated state. This was recognised by Bakker-Grunwald and Van Dam [13, 22], who studied this phenomenon in some depth. They concluded that the slow kinetics on the first illumination reflected the slow modulation of CF_0-CF_1 by dithiothreitol. However, they also concluded that once completed, thiol modulation (in broken chloroplasts) was essentially irreversible. Thus, the more rapid appearance of ATPase activity on the second illumination reflected reactivation of thiol-modulated complexes that had undergone pH deactivation in the dark in the absence of $\Delta\bar{\mu}_H{}^+$ [13,22].

Our observations with intact chloroplasts are also shown in Fig. 1. The rate of appearance of activity is fast on both the first and second illuminations, suggesting that thiol modulation is less rate limiting in the intact organelle. This would be the case if the concentration of thioredoxin in the stroma is relatively high, since the rate of modulation is proportional to the thioredoxin concentration [21]. We also observe that ATPase activity declines to the dark-adapted level within 7 min of terminating illumination of intact chloroplasts. In order to determine whether this decline was caused by thiol demodulation, we performed the following experiment. Intact chloroplasts were illuminated to induce maximal thiol modulation of CF_0-CF_1, then lysed into media containing 5 mM dithiothreitol. The presence of dithiothreitol should prevent any subsequent thiol demodulation [13,22]. (In fact, dithiothreitol reportedly

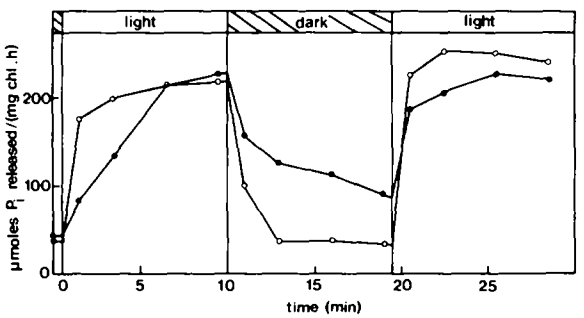

Fig. 1. ATPase activity observed during a light-dark-light regime. Chloroplasts were either intact (o———o) or lysed (•———•) in the activation stage. Dithiothreitol (2 mM) and methyl viologen (100 µM) were added to lysed chloroplasts but not to intact organelles.

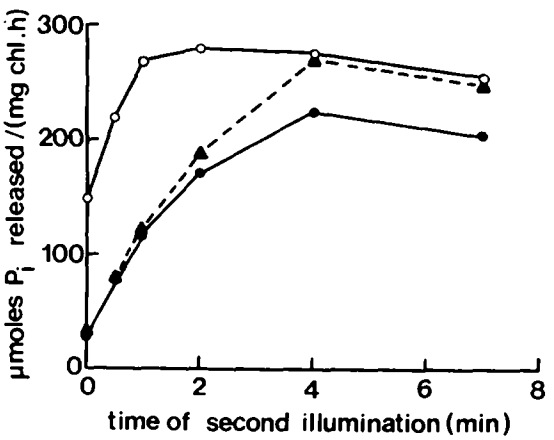

Fig. 2. Kinetics of appearance of ATPase activity on the second illumination. Intact chloroplasts were illuminated for 2 min then lysed in the activation stage with media containing dithiothreitol (final concentration 5 mM). A total of 8 min darkness was allowed before commencing the second illumination. All samples contained 100 µM methyl viologen. (o———o) Chloroplasts were lysed immediately after terminating preillumination; (•———•) chloroplasts were intact for 7 min in darkness before lysis; (▲- - - - -▲) no preillumination was given and chloroplasts were lysed in the dark.

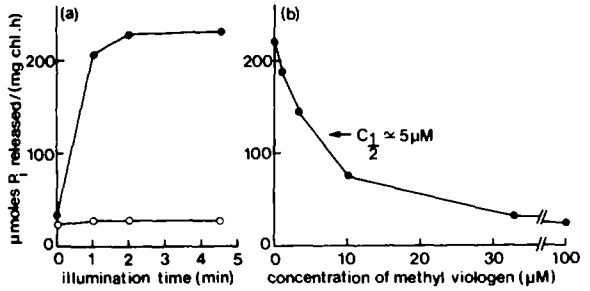

Fig. 3. Effect of methyl viologen on thiol modulation of CF_0-CF_1 in intact chloroplasts. (a) Dark-adapted intact chloroplasts were illuminated in the presence (○———○) or absence (●———●) of 200 µM methyl viologen in the activation stage; (b) dependence of observed activity on the concentration of methyl viologen in the activation stage (illumination time 4 min).

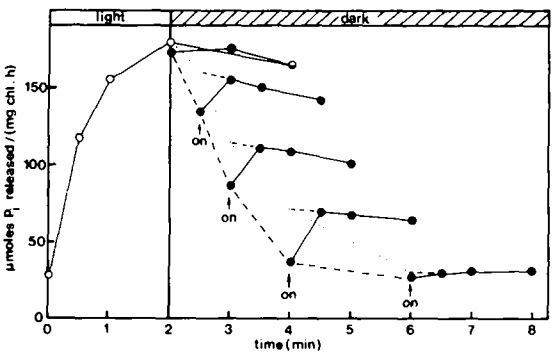

Fig. 4. Thiol demodulation of CF_0-CF_1 in intact chloroplasts in the dark. Intact chloroplasts were illuminated for 2 min then allowed various dark intervals before being reilluminated. 15 s prior to reillumination, 100 µM methyl viologen was added. Each reillumination represents a separate experiment. The dashed line connects the activities observed immediately before reillumination, and thus represents the decline in total activity. The dotted line connects the (projected) activities of pH-reactivated chloroplasts at the time of reillumination, and thus represents the rate at which thiol demodulation of CF_0-CF_1 proceeds in darkness,

modulates CF_0-CF_1 very slowly even in darkness [26], but this effect does not seem to occur under the conditions used here (results not shown).) Upon reilluminating the (now broken) chloroplasts, the rate of appearance of ATPase activity will be rapid if CF_0-CF_1 complexes are thiol modulated, and slow if they are not. As shown in Fig. 2, chloroplasts which were lysed into dithiothreitol immediately following illumination show, upon reillumination, the rapid appearance of ATPase activity that is characteristic of thiol-modulated CF_0-CF_1 complexes. In contrast, chloroplasts which were illuminated and then left in the dark for 7 min prior to lysis show the slow appearance of ATPase activity that is observed with completely dark-adapted chloroplasts (Fig. 2). THus, it appears that thiol modulation of CF_0-CF_1 has been reversed in intact chloroplasts during the 7 min dark interval between preillumination and lysis.

In order to confirm this result, we made use of an effect of methyl viologen briefly noted earlier [21]. As shown in Fig. 3a, 200 µM methyl viologen completely prevents the appearance of ATPase activity normally observed after illuminating dark-adapted, intact chloroplasts. Providing that catalase is present, the concentration at which methyl viologen inhibits the appearance of ATPase activity by 50% is 5 µM (Fig. 3b), which coincides well with its affinity as an electron acceptor for PS I. Since viologens do not inhibit the pH activation of CF_0-CF_1 [15], the inhibition must be ascribed to interference at the thiol-modulation step. Moreover, since methyl viologen does not inhibit thiol modulation dependent on dithiothreitol (Figs. 1 and 2), its inhibition in intact chloroplasts must be due specifically to interference with the thioredoxin system. This could be due to production of oxidising radicals or H_2O_2 [9]. However, in the presence of catalase it is more likely that methyl viologen simply acts as a more efficient electron acceptor for PS I than ferredoxin, preventing net reduction of thioredoxin. Similar effects have been observed using other electron acceptors, such as phenazine methosulphate [21,27] and oxaloacetate [27] with intact chloroplasts, and $NADP^+$ with broken chloroplasts reconstituted with the thioredoxin system [21].

The ability of methyl viologen to block thiol modulation (but not pH activation) of CF_0-CF_1 in intact chloroplasts has been utilised to study the reversal of these processes in darkened organelles. Fig. 4 shows the results of an experiment where intact chloroplasts were illuminated to induce maximal pH activation and thiol modulation of CF_0-CF_1, then left for various dark intervals before a second illumination was given. Just prior to reillumination, 100 µM methyl viologen was added to prevent any subsequent thiol modulation in the light. After 7 min darkness (Fig. 4), reillumination of intact chloroplasts plus methyl viologen produced little observable

ATPase activity. This result again suggests that thiol demodulation of CF_0-CF_1 had largely taken place during the 7 min dark interval. After shorter dark intervals (Fig. 4), reillumination partially restored ATPase activity. This restoration can be ascribed to pH reactivation of thiol-modulated CF_0-CF_1 complexes that had deactivated in the dark. Finally, when methyl viologen was added in the light (Fig. 4), there was little change in ATPase activity during the subsequent 2 min of illumination, although activity was thereafter observed to decline (results not shown). The latter observation shows that methyl viologen does not promote rapid thiol demodulation in the light as would be expected if oxidants generated during its photoreduction were responsible for its inhibitory effects. This observation is the basis for the earlier assertion that methyl viologen blocks thiol modulation of CF_0-CF_1 simply by competing for electrons from PS I and preventing net reduction of thioredoxin.

Thus, the data of Fig. 4 indicate that the decline in total ATPase activity (dashed line) in intact chloroplasts in the dark can be ascribed to two processes: (a) pH deactivation, and (b) thiol demodulation of CF_0-CF_1. Reillumination (plus methyl viologen) reverses a but not b. The rate at which thiol demodulation occurs is thus given by the dotted line in Fig. 4, and in this experiment the process is seen to be complete within 5 min darkness. Data from a number of experiments of this type (and of those depicted in Fig. 2) indicated that, at 20°C, thiol demodulation of CF_0-CF_1 in intact pea chloroplasts is invariably completed within 10 min of terminating illumination.

Thiol demodulation of CF_0-CF_1 in lysed chloroplasts

The use of methyl viologen to block thiol modulation of CF_0-CF_1 in intact chloroplasts can be extended to study thiol demodulation in lysed chloroplasts. Fig. 5 shows the decline in total activity observed after chloroplasts had been illuminated intact then lysed into media which did not contain dithiothreitol. For comparison, a similar experiment is shown where the intact chloroplasts were simply diluted and not lysed. Using pea chloroplasts (variety Feltham First), we have invariably observed that the rate of decline of activity in the dark in intact chloroplasts is equal to, or faster than, that in lysed chloroplasts. This result is in contradiction to that observed by Mills and Hind [19] (recently confirmed by

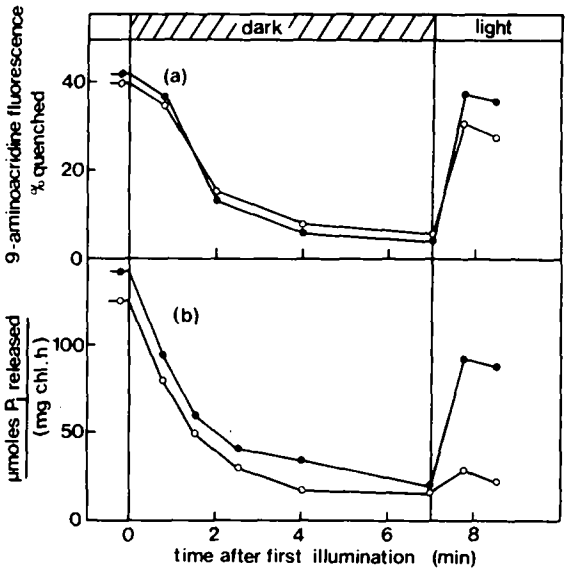

Fig. 5. Comparison of the decline in total activity and the thiol demodulation of CF_0-CF_1 in intact and lysed chloroplasts. Intact chloroplasts (approx. 0.7 mg Chl/ml) in 0.36 M sorbitol, 30 mM Tricine, catalase (2000 U/ml), pH 8.0, were illuminated for 3 min and then lysed by osmotic shock or simply diluted with isotonic medium. In either case, the final medium contained 5 mM $MgCl_2$ and 100 μM methyl viologen. Reillumination was carried out after 7 min darkness. (a) ATP-dependent 9-aminoacridine fluorescence quenched was estimated by comparing the quenched level with the level of fluorescence observed after adding 10 mM NH_4Cl. The quenching thus includes a small percentage due to dark quenching processes that are independent of ATP but are released by adding NH_4Cl. (b) P_i release. (o———o) Intact; (•———•) lysed in the dark.

Schreiber [28]), using spinach chloroplasts. The latter results indicate that ATPase activity is more stable in intact spinach chloroplasts than in lysed organelles [19,28]. The discrepancy is not due to differences in experimental methodology, since essentially similar results were obtained by observing ATP-dependent 9-aminoacridine fluorescence quenching (Fig. 5a) or P_i release (Fig. 5b). Possible explanations for these differing results are given in the Discussion.

Fig. 5b shows the results of a second period of illumination after 100 μM methyl viologen had been added in the dark interval. It can be seen that CF_0-CF_1 in chloroplasts which were lysed in the dark undergoes considerable reactivation when illuminated, whereas much less reactivation is seen with chloroplasts maintained intact in the dark. This result

is also observed with ATP-dependent 9-aminoacridine fluorescence quenching (Fig. 5a) but appears to be less conclusive. However, it has been shown that 9-aminoacridine fluorescence quenching saturates at relatively low levels of coupled ATP hydrolysis [13,22], possibly due to the 'gating' function of CF_0-CF_1 [15]. The quenching of 9-aminoacridine fluorescence is thus not a reliable quantitative measure of the rate of ATP hydrolysis. We also observed (results not shown) that the levels of quenching were stable when chloroplasts were lysed, but unstable when chloroplasts were intact in the dark interval. The inability to maintain a stable level of quenching is indicative of a lower rate of ATP hydrolysis [13, 22], and thus is in agreement with the more quantitative studies of ATP hydrolysis of Fig. 5b.

The results of Fig. 5 indicate that thiol demodulation of CF_0-CF_1 occurs readily in intact chloroplasts but not after they are lysed. The intact organelle appears therefore to possess a system (presumably in solution in the stroma) that actively reverses the thiol modification of CF_0-CF_1. Studies with other thiol-regulated stromal enzymes have suggested several oxidants that might promote the thiol-demodulation process [5,6,9,10,29], and the experimental conditions of Fig. 5 provide a means of testing their effects on thiol-modulated CF_0-CF_1.

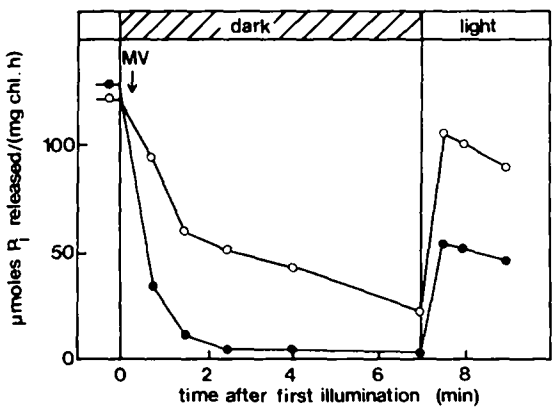

Fig. 6. Effect of NaN_3 and H_2O_2 on the decline of ATPase activity and the thiol demodulation of CF_0-CF_1 in lysed chloroplasts in the dark. Experiments were carried out essentially as described in the legend to Fig. 5 except both samples were lysed at the termination of preillumination. (o———o) Control; (•———•) NaN_3 and H_2O_2 were added to give a final concentration of 0.5 mM and 94 µM, respectively. MV, methyl viologen.

TABLE I

EFFECT OF OXIDANTS ON THE THIOL DEMODULATION OF CF_0-CF_1 IN LYSED CHLOROPLASTS

Experiments were performed as shown in Fig. 6. Intact chloroplasts were illuminated for 2 min, then lysed and 100 µM methyl viologen added. After 7 min darkness, 30 s of reillumination was given and the noted activities were observed. Oxidants were added as indicated at the time of lysis.

Oxidant added (final concentration)	ATPase activity observed after 30 s reillumination (µmol P_i released/mg Chl per h)
None	101
Oxidised glutathione (28 mM)	96
$Thioredoxin_f$ (27 µg/ml)	79
$K_3Fe(CN)_6$ (1 mM)	13

Fig. 6 shows the effect of adding H_2O_2 (plus 0.5 mM azide to inhibit catalase) immediately after lysis of preilluminated chloroplasts. H_2O_2 dramatically increases the rate of decline of activity in the dark and promotes thiol demodulation as seen by the lower levels of activity observed after the second illumination. Table I lists several other oxidants tested in this way. Oxidised glutathione (28 mM) had only minor effects and $thioredoxin_f$ (at a concentration of approx. 2 µM) was only slightly more effective (see Discussion). The inorganic oxidant ferricyanide was extremely effective, and promoted complete thiol demodulation of CF_0-CF_1 within the 7 min dark interval.

Effect of temperature on thiol demodulation in intact and lysed chloroplasts

We have observed that the process responsible for thiol demodulation of CF_0-CF_1 in intact chloroplasts is quite temperature sensitive. Fig. 7 shows that lowering the temperature to 3.5°C prior to ending illumination greatly stabilises the pH-activated, thiol-modulated state of CF_0-CF_1 in the intact organelle. Even after 27 min darkness at 3.5°C, significant activity is observed on reillumination, indicating that both the pH-deactivation and the thiol-demodulation processes are retarded at lower temperatures.

As shown in Table II, intact chloroplasts that had been illuminated, then lysed and stored on ice, also

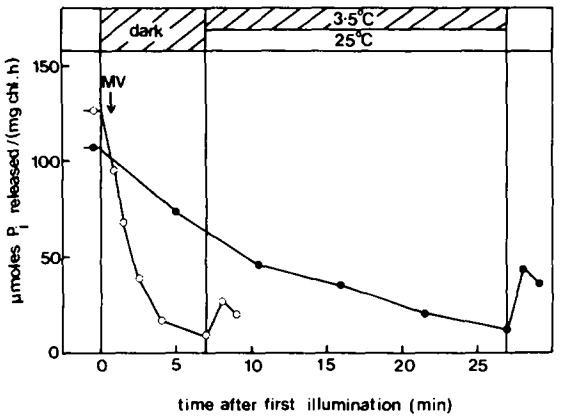

Fig. 7. Effect of temperature on the decline of total activity and the thiol demodulation of CF_0-CF_1 in intact chloroplasts in the dark. Experiments were carried out essentially as described in the legend to Fig. 5 except that chloroplasts were diluted and not lysed at the end of the preillumination. 1 min before terminating preillumination, the activation stage was placed in a stirred ice-water bath through which illumination was continued. 1 min after the second illumination commenced, the ice-water bath was removed and the temperature returned to 20°C. Temperature equilibration (both raising and lowering) was complete within 1 min. (●——●) 3.5°C; (○——○) 25°C. MV, methyl viologen.

TABLE II

THE DECLINE OF ATPase ACTIVITY AND THE THIOL DEMODULATION OF CF_0-CF_1 IN LYSED CHLOROPLASTS AT 4°C

Intact chloroplasts (Pea, variety Meteor) were illuminated for 3 min at 20°C, then lysed into media not containing dithiothreitol. 100 μM methyl viologen was added, and the chloroplasts were placed on ice in the dark. At the indicated times, samples were removed and assayed for ATPase activity either with or without giving them a second period of illumination (30 s) to reactivate CF_0-CF_1. The activity immediately following the first illumination was observed to be 153 μmol P_i released/mg Chl per h.

Time of storage in the dark following the first illumination (min)	ATPase activity observed (μmol P_i released/ mg Chl per h)	
	Without reillumination	With reillumination
20	113	146
40	59	117
60	37	93
80	22	72
100	17	65
120	7	57

maintain high levels of ATPase activity for long periods in the dark. The activity observed after a short reillumination indicates that thiol demodulation is extremely slow under these conditions, as has been observed with broken chloroplasts that have been thiol-modulated with dithiothreitol [13,16]. Unlike some authors [13], we did not observe that the thiol-modulated state of CF_0-CF_1 was completely stable, but the time required for approx. 50% thiol demodulation was 1–2 h for lysed chloroplasts stored on ice in the dark. Thus, illumination and lysis of intact chloroplasts should prove to be a useful method for preparing broken chloroplasts possessing thiol-modulated CF_0-CF_1 without the need to add or remove exogenous dithiols.

Discussion

The major conclusion to be drawn from these studies is that reversal of the light-dependent thiol modulation of CF_0-CF_1 occurs readily in darkened intact chloroplasts but not when the organelles are lysed. This situation resembles the forward modulation process which also is greatly decreased in extent and rate after lysis of intact chloroplasts [19,20], presumably because of dilution of the thioredoxin system (or other chloroplast dithiols [4]). Similarly, the intact organelle must possess a soluble or loosely bound system that promotes reversal of thiol modulation of CF_0-CF_1. The simplest such system would be a pool of oxidant that opposes the reductive effects of reduced thioredoxin. Glutathione has been shown to reverse thiol modulation of fructose bisphosphatase [5], and oxidised thioredoxin itself has also been proposed to be a physiological oxidant [29]. In the case of CF_0-CF_1, oxidised glutathione appears to be relatively ineffective at quite high concentrations and is unlikely to be the physiological oxidant unless a special glutathione CF_0-CF_1 oxidoreductase exists. Thioredoxin was only partially effective at a concentration of 2 μM, but there are some indications that the stromal concentration of this cofactor is higher [20]. Any definite conclusions in respect of the thioredoxin system as the active oxidant for CF_0-CF_1 must await further experiments on its redox properties and requires some information on its physiological concentration.

Other oxidants may also be important in the chloroplast, including H_2O_2. However, the concentration

of H_2O_2 that stimulates thiol demodulation of CF_0-CF_1 would also inhibit its modulation in the light [28], as well as the modulation of other stromal enzymes [7–9]. One would have to assume therefore that the steady-state concentration of H_2O_2 is higher in the dark than in the light. Such an assumption is initially unappealing, because a major source of H_2O_2 must be from reduction of O_2 at PS I, a light-dependent process [7].

The overall stability of ATPase activity in the dark is thus a complex function of the rate of pH deactivation (dependent on the levels of ADP, ATP and P_i [12,30,31] and the rate of thiol demodulation (dependent on the thioredoxin system, stromal oxidants and possibly a specific redox poise between the two). It is therefore not surprising that the overall rate of decay of total ATPase activity in the dark may vary according to such conditions as chloroplast integrity, growth conditions, etc., and thus give rise to apparent differences between different preparations (for example, spinach [19] and pea chloroplasts noted earlier).

Finally, these studies, together with those of Marchant [32] may provide an explanation for some earlier reports that dark-adapted chloroplasts show significant [30,31] or high rates of CF_0-CF_1 ATPase activity [33]. In all cases, the chloroplasts were not intact as judged by the permeability of their envelopes to adenine nucleotides, though in one case they were described as 'class I' [33]. Marchant [32] has shown that preillumination of spinach leaves for 30 min at 4°C followed by rapid isolation of chloroplasts gives rise to broken chloroplasts with partially thiol-modified CF_0-CF_1 complexes. The data presented here indicate that in leaves or intact chloroplasts the effects of preillumination would be rapidly reversed within a few minutes at room temperature, but would be much more stable at 3.5°C. Thus, if preillumination were carried out at lower temperatures, which is a standard method in the isolation of chloroplasts, or if chloroplast isolation were carried out in bright light, a proportion of the resulting preparation of broken chloroplasts might be thiol modulated in respect of CF_0-CF_1 and hence would give higher rates of ATP hydrolysis in the absence of reduced dithiols, especially after a brief illumination to pH activate CF_0-CF_1. Care must therefore be exercised before ascribing such activity to non-thiol-modulated CF_0-CF_1 [33].

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