Role of the \(\gamma\) Subunit of Chloroplast Coupling Factor 1 in the Light-dependent Activation of Photophosphorylation and ATPase Activity by Dithiothreitol*

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In leaves and intact chloroplasts, oxidation and reduction have been shown previously to regulate the ATPase activity of thylakoids. Illumination of spinach chloroplast thylakoids in the presence of dithiothreitol, which activates the ability of thylakoids to catalyze sustained ATP hydrolysis in the dark, causes increased incorporation of N-ethylmaleimide into the \(\gamma\) subunit of coupling factor 1 (CF1). A disulfide bond in the \(\gamma\) subunit is reduced during activation. The residues involved in this disulfide bond are the same as those in the disulfide linkage reduced during dithiothreitol activation of soluble CF1. The disulfide and dithiol forms of the \(\gamma\) subunit may be separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. N-Ethylmaleimide is preferentially incorporated in the dark into the reduced form of the \(\gamma\) subunit of CF1 in thylakoids previously exposed to dithiothreitol. Only a subpopulation of the CF1 in thylakoids is susceptible to dithiothreitol reduction and subsequent reaction with N-ethylmaleimide in the dark. Alkylation of the thiol groups exposed by reduction of the disulfide bond protects ATPase activity from inhibition by oxidants. At a given value of the transmembrane pH differential, photophosphorylation rates in dithiothreitol-activated thylakoids can be as much as seven to eight times those of nonactivated controls. N-Ethylmaleimide treatment of activated thylakoids in the dark prevents the loss of the stimulation of ATP synthesis on storage of the thylakoids. Photophosphorylation by intact chloroplast lysed in assay mixtures is also activated in comparison to that by washed thylakoids. At a low ADP concentration, the rate of photophosphorylation approaches saturation as \(\Delta pH\) increases. These results suggest that the \(\gamma\) subunit of CF1 plays an important role in regulation of ATP synthesis and hydrolysis.

One of the peculiar features of washed chloroplast thylakoid membranes is that they catalyze negligible rates of ATP hydrolysis in the dark, even though they catalyze very high rates of ATP synthesis in the light. The treatment of thylakoids in the light with trypsin (1, 2) or a thiol compound (3), such as dithiothreitol, activates the ability of thylakoids to hydrolyze ATP in the dark. This ATPase activity and photophosphorylation are both inhibited by several inhibitors of the ATPase activity of soluble CF1, including an antiserum to CF1 (4, 5). Furthermore, the ATPase activity of thylakoids is coupled to proton translocation into the thylakoids (6), while ATP synthesis is coupled to proton translocation out of the thylakoids. These observations indicate that the ATP hydrolysis and synthesis activities of thylakoids are catalyzed by the same enzyme and must share at least some common steps in their mechanisms.

Activation may be divided experimentally into two energy-dependent phases (7). The ATPase activity of thylakoids previously illuminated in the presence of dithiothreitol decreases as the time interval between the end of activating illumination and the addition of ATP increases. This dark decay of the ATPase activity is remarkably accelerated by ADP, but is slowed by ATP and P, (5). After the ATPase activity has decayed in the dark, activity may be restored by illumination of thylakoids in the absence of dithiothreitol (7). Both phases of activation are sensitive to uncouplers.

Since CF1 extracted from the treated membranes is an active ATPase (4), the energy- and dithiothreitol-dependent phase of activation modifies CF1. However, this modification of CF1 by dithiothreitol is not sufficient to allow expression of its membrane-bound ATPase activity, as shown by decay of this activity in the dark. Energy-dependent conformational changes that result in release of tightly bound ADP (8, 9), and perhaps other energy-dependent alterations, are also required.

CF1, in intact chloroplasts (10–12) and leaves (13, 14) can be activated to a state similar to that of CF1 in thylakoids illuminated in the presence of dithiothreitol. Illumination of intact chloroplasts in the absence of added reductant activates ATPase activity, assayed in the dark after lysis of the chloroplasts. A thioredoxin system probably provides reducing equivalents for this activation in intact chloroplasts (15). Furthermore, there appears to be an oxidizing system in intact chloroplasts which deactivates CF1 in the dark, further suggesting that reduction and oxidation of CF1 may be an important form of regulation in vivo (11).

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Because the illumination of thylakoids under reducing conditions activates ATP hydrolysis, thermodynamic considerations suggest that the same treatment should also activate ATP synthesis. There are several indications that this is the case. ATP formation in the dark occurs at lower artificially imposed ΔpH values in thylakoids previously illuminated in the presence of dithiothreitol than in control thylakoids (16). Moreover, photophosphorylation at a given ΔpH value is higher in light- and dithiothreitol-activated thylakoids than in nonactivated ones (17). Dithiothreitol treatment of soluble CF$_1$, which activates its ATPase activity (4) and reduces a disulfide bond in its γ subunit (18, 19), enhances the ability of CF$_1$ to stimulate ATP synthesis in CF$_1$-depleted thylakoids (20). Light and dithiothreitol treatment of thylakoids also allows ATP synthesis to begin after fewer flashes of light (21).

The γ subunit of CF$_1$ contains four cysteinyl residues, distinguished by their varying ability to react with alkylating reagents and by their chromatographic separation following trypsin digestion of the γ subunit (19, 22). The same accessible sulfhydryl (S4) is labeled by treating oxidized or reduced soluble CF$_1$, with alkylating reagents or by treating nonactivated or dithiothreitol-activated thylakoids with alkylating reagents in the dark or light. The same buried sulfhydryl (S3) is labeled by treating oxidized or reduced soluble CF$_1$, with alkylating reagents in the presence of a 1% sodium dodecyl sulfate or by trypsin in thylakoids previously nonactivated or dithiothreitol-activated thylakoids with alkylating reagents in the light, but not in the dark. Two cysteinyl residues form a disulfide bond in oxidized CF$_1$ (S1 and S2) and are labeled by treating reduced, but not oxidized, soluble CF$_1$, with alkylating reagents.

In this communication, we report that the activation of ATP hydrolysis by dithiothreitol treatment of thylakoids reduces the same disulfide linkage in the γ subunit of CF$_1$, that is reduced by dithiothreitol treatment of soluble CF$_1$. Alkylation of these thiol groups with N-ethylmaleimide remarkably protects the activated form of the membrane-bound enzyme from inactivation by oxidants. Reduction of the disulfide also activates photophosphorylation. The possible nature of this activation is discussed.

**MATERIALS AND METHODS**

Spinach chloroplast thylakoids (23) and intact (>85%) pea chloroplasts (24) were prepared as described. Unless otherwise stated, the illumination (2 × 10$^6$ erg cm$^{-2}$ s$^{-1}$) of thylakoids (0.1 mg of chlorophyll cm$^{-2}$) in the presence of 5 mM dithiothreitol was carried out at 20 °C in an incubation mixture that contained 20 to 50 mM Tricine-NaOH (pH 8.0), 50 mM NaCl, 5 mM MgCl$_2$, and 0.010 to 0.025 mM pyocyanine. In most experiments, the thylakoids were collected by centrifugation at 3000 × g for 7 min at 0-4 °C, resuspended to about 0.1 mg of chlorophyll ml$^{-1}$ in a cold, buffered sucrose solution (0.4 M sucrose, 0.02M Tricine-NaOH (pH 8.0), and 0.01 M NaCl) and recollected by centrifugation. The pellets were reuspended in a minimal volume of the buffered sucrose solution.

Photophosphorylation with pyocyanine as the mediator of electron flow was determined at various light intensities (0.1 to 5 × 10$^6$ erg cm$^{-2}$ s$^{-1}$) in reaction mixtures that contained 23 mM Tricine-NaOH (pH 8), 50 mM NaCl, 5 mM MgCl$_2$, 0.02 mM pyocyanine, 3 or 5 mM potassium phosphate buffer (pH 8.0) containing about 5 × 10$^{-5}$ M of [32P]ADP, either a limiting (0.01 mM) or saturating (0.5 mM) ADP concentration, and thylakoids equivalent to 0.1 to 0.2 mg of chlorophyll per milligram chlorophyll. Since 32P was determined either by an extrac-
avoid the use of the large quantities of [3H]N-ethylmaleimide necessary for optimal labeling in illuminated thylakoids. Thylakoids were treated in the light with diethiothreitol, pelleted, resuspended, and incubated with 2 mM N-ethylmaleimide in the light or dark (35), and CFI was isolated. Aliquots of the CFI preparations were desalted, denatured by boiling for 1 min in the presence of 1% sodium dodecyl sulfate, and incubated with 9 mM [3H]N-ethylmaleimide (90,000 cpm/mmol) for 14 min at room temperature. Unreacted [3H]N-ethylmaleimide was removed and aliquots were subjected to electrophoresis. The specific activity of [3H]N-ethylmaleimide was estimated as described in the preceding report (22). The molecular weight of CFI was assumed to be 400,000 (37) in the calculation of [3H]N-ethylmaleimide incorporation.

RESULTS

Effect of Illumination in the Presence of Dithiothreitol on the Modification of Sulfhydryl Groups of CFI.—Since illumination of thylakoids in the presence of diethiothreitol induces a Ca2+-dependent ATPase activity in CFI extracted from the thylakoids with EDTA (4), this treatment clearly alters CFI. The disulfide bond in CFI, present in the γ subunit of the soluble enzyme (18, 19), may be cleaved by this treatment and support for this prediction is shown in Table I. Thylakoids were incubated with N-ethylmaleimide in the dark, followed by incubation with diethiothreitol in the dark or the light. The sulfhydryl compound was removed by washing and the thylakoids were incubated with [3H]N-ethylmaleimide in the dark. The [3H]N-ethylmaleimide was incorporated almost exclusively into the γ subunit, indicating that a disulfide bond in this subunit was cleaved. Some incorporation of [3H]N-ethylmaleimide into the γ subunit of thylakoids exposed to diethiothreitol in the dark was also detected. Since the accessible thiol (S4) on the γ subunit is blocked by pretreatment with nonradioactive N-ethylmaleimide (35), this incorporation of radioactive maleimide into the γ subunit is probably the result of limited cleavage of a disulfide. This conclusion is consistent with the observation that CFI, extracted from thylakoids incubated with diethiothreitol in the dark has some ATPase activity (4). The extents of activation and maleimide modification are both enhanced by illumination.

Slightly less than 1 mol of maleimide/mol of CFI was incorporated into the γ subunit of CFI in thylakoids previously illuminated in the presence of diethiothreitol. If both of the thiols formed by the reduction of a disulfide bond were accessible to N-ethylmaleimide, the maximal incorporation of maleimide would be 2/CFI. Incomplete reaction could result from the inaccessibility of one thiol to reaction with the alkylating reagent, reduction of the disulfide linkage of only about half of the CFI, or reoxidation prior to treatment with labeled maleimide. Partial reduction appears to explain, at least in part, the incomplete incorporation of [3H]N-ethylmaleimide. The γ subunit of reduced and maleimide-modified CFI, migrates as two components in sodium dodecyl sulfate-polyacrylamide gel electrophoresis if CFI is not pretreated with diethiothreitol immediately before electrophoresis (Fig. 1, Lanes 2 and 4). The γ subunit of the diethiothreitol-pretreated enzyme migrates as a single component with a mobility identical to the slower moving component (Fig. 1, Lanes 5 and 6). Thus, the γ component that migrates faster is probably the disulfide form, whereas the more slowly migrating component is probably the reduced and alkylated form. Alkylation prevents formation of the disulfide, even in the presence of o-iodosobenzoate (Fig. 1, Lane 2), a reagent that oxidizes sulfhydryls to disulfides. In agreement with this conclusion, the more slowly moving component contains 87% of the [3H]N-ethylmaleimide incorporated into the γ subunit (Table II).

![Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of reduced and oxidized CFI.](image)

**TABLE I**

Incorporation of [3H]N-ethylmaleimide into CFI, in thylakoids in the dark following diethiothreitol treatment in the dark or light

| Thylakoids, previously treated with 2 mM N-ethylmaleimide in the dark, were either illuminated or kept in the dark in the presence of 5 mM diethiothreitol. The thiol compound was removed by washing and the thylakoids were incubated with 0.25 mM [3H]N-ethylmaleimide (6 × 10⁴ cpm/mmol) for 5 min in the dark at room temperature. Diethiothreitol was added to a final concentration of 0.28 mM and CFI was isolated. Duplicate aliquots (9 μg) of each CFI preparation were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. After staining the gel, the radioactivity in each band was determined. |
|---|---|---|---|
| Subunit | Treatment | Dithiothreitol in dark | Dithiothreitol in light |
|---|---|---|---|
| α | mol [3H]N-ethylmaleimide/mol CFI | 0.01 | 0.03 |
| β |  | 0.02 | 0.04 |
| γ |  | 0.23 | 0.92 |
| δ | <0.01 | 0.01 | 0.01 |
| ε | <0.01 | 0.01 | 0.01 |

**TABLE II**

Distribution of [3H]N-ethylmaleimide and protein between the two components of the γ subunit of CFI, alkylated in the dark in thylakoids after diethiothreitol treatment

| Thylakoid treatment | Pretreatment before electrophoresis | [3H]NEM | Protein |
|---|---|---|---|
| | | γ1 | γ2 |
| | | | % |
| | | | |
| DTT in dark | DTT | 97 | 3 | 100 | 0 |
| None | 88 | 12 | 77 ± 5 | 83 ± 5 |
| IBZ | 87 | 13 | 23 ± 5 | 77 ± 5 |
| DTT in light | DTT | 96 | 4 | 100 | 0 |
| None | 96 | 4 | 64 ± 10 | 36 ± 10 |
| IBZ | 89 | 11 | 63 ± 10 | 37 ± 10 |
Although it is difficult to obtain a precise estimate because of limits to the resolution of densitometry, approximately 60\% of the $\gamma$ subunit of the CF$_1$ from thylakoids alkylated after illumination in the presence of diethiothreitol was in the slower migrating band (Fig. 1, Lanes 2 and 4), indicating reduction of the disulfide bond in 60\% of the CF$_1$. Only about 20\% of the $\gamma$ subunit of the CF$_1$ from thylakoids alkylated after dark incubation with diethiothreitol was in the slower migrating band. Omitting diethiothreitol pretreatment before electrophoresis does not change the amount of protein in the $\alpha$ or $\beta$ bands or in the sum of the $\gamma1$ and $\gamma2$ bands (Fig. 1). Moreover, no new bands appear, suggesting that there are no inter-subunit disulfide bonds in CF$_1$.

Diethiothreitol activates the ATPase activity of soluble CF$_1$ and cleaves the disulfide ($S1$ and $S2$) (18, 19). As shown in Fig. 2, diethiothreitol treatment of soluble and bound CF$_1$ reduces the same disulfide. Thylakoids were treated with N-ethylmaleimide in the dark, activated, and then labeled with radioactive N-ethylmaleimide in the dark. CF$_1$ was purified and cleaved with trypsin, and cysteine peptides were separated by HPLC (22). Only two major peptides were labeled, $S1$ and $S2$, the same peptides shown to participate in the disulfide linkage in the soluble enzyme (22). Similar results were obtained with the enzyme from activated thylakoids labeled in the dark with 4-vinylpyridine (not shown).

The illumination of thylakoids, pretreated with N-ethylmaleimide in the dark, in the presence of $[^3H]$N-ethylmaleimide causes a partial inhibition of photophosphorylation and incorporation of $[^3H]$N-ethylmaleimide specifically into a sulfhydryl ($S3$) of the $\gamma$ subunit (22, 35). The incomplete nature of both the inhibition and extent of incorporation has been a vexing problem. Usually, the maximal inhibition of photophosphorylation is about 70\% and up to 0.7 mol of maleimide is incorporated per mol of CF$_1$. Prior illumination of thylakoids in the presence of diethiothreitol has no effect on the ability of N-ethylmaleimide to inhibit photophosphorylation (data shown below). However, whatever is limiting reduction of the disulfide in the $\gamma$ subunit of CF$_1$ in thylakoids may also be limiting alkylation of the buried sulfhydryl of the $\gamma$ subunit. This explanation may be at least partially correct. Thylakoids were illuminated in the presence of diethiothreitol and then incubated with N-ethylmaleimide either in the dark or in the light. In both thylakoid preparations, N-ethylmaleimide should have reacted with the accessible sulfhydryls of the $\gamma$ and $\epsilon$ subunits and with the sulfhydryls formed by the partial reduction of the disulfide. The buried sulfhydryl of the $\gamma$ subunit of those thylakoids incubated in the light should have been partially blocked. CF$_1$ was then isolated from both preparations and incubated with $[^3H]$N-ethylmaleimide in the presence of sodium dodecyl sulfate. Only thiols in the $\alpha$ and $\beta$ subunits and the unprotected fraction of the buried thiol of the $\gamma$ subunit should be labeled. Illumination of activated thylakoids in the presence of N-ethylmaleimide had no effect on the incorporation of $[^3H]$N-ethylmaleimide into the oxidized form of the $\gamma$ subunit, whereas it inhibited $[^3H]$N-ethylmaleimide incorporation into the reduced form by about 30\% (Table III). Thus, those CF$_1$ molecules that were not reduced in thylakoids were also not alkylated at the buried sulfhydryl. This suggests that alkylation of the buried sulfhydryl in thylakoids and reduction of the disulfide are limited by a common factor. For example, some of the CF$_1$ might be bound to thylakoids unable to maintain a $\Delta\psi$ sufficient to promote energy-dependent reduction or alkylation.

Effect of Maleimides on Deactivation.—After light and diethiothreitol treatment and removal of most of the diethiothreitol, thylakoids can retain for up to several hours their ability to hydrolyze ATP in the dark, provided they are briefly illuminated prior to assay (7). However, the decay of light-induced Mg$^{2+}$-ATPase activity in thoroughly washed and homogenized thylakoids, while variable, can be complete within less than 30 min. When rapid decay is observed, Mg$^{2+}$-ATPase activity may be partially reactivated by illumination in the presence of diethiothreitol (Table IV). The decay in the ability of activated thylakoids to hydrolyze ATP after a second illumination is probably the result of oxidation of the diethiothiol groups in the $\gamma$ subunit to a disulfide. Oxidation may be enhanced by aeration during homogenization or by the presence of trace amounts of heavy metal cations. A variability in the heavy metal content of thylakoid suspensions may explain this.

![Image](image-url)

**Fig. 2.** Separation of peptides containing $[^3H]$N-ethylmaleimide by HPLC. CF$_1$ was isolated from thylakoids first treated with N-ethylmaleimide in the dark, then activated by illumination in the presence of diethiothreitol, and finally reacted with $[^3H]$N-ethylmaleimide in the dark. An aliquot containing 350 pg of protein and 4400 cpm of $[^3H]$N-ethylmaleimide was digested with trypsin and the peptides were separated by HPLC. Two major fractions were collected and 1-ml aliquots were taken for determination of radioactivity. A background radioactivity of 25 cpm was subtracted from each sample. Expected elution times for the cysteinyl-containing peptides are indicated by arrows and were obtained from Ref. 22: the elution times for $S3$ and $S4$, labeled in thylakoids with N-ethylmaleimide, were taken directly from Fig. 5 of Ref. 22, while 8 min was added to the elution times in Fig. 6 of Ref. 22 for $S1$ and $S2$, labeled in soluble CF$_1$ with vinylpyridine, to take into account the effect of modifying reagent on elution time (22).

**Table III**

**Preferential incorporation of N-ethylmaleimide into reduced CF$_1$ in illuminated thylakoids**

| Component | $[^3H]$N-ethylmaleimide incorporation |
|-----------|-------------------------------------|
|           | NEM-dark CF$_1$ | NEM-light CF$_1$ | Dark-light |
| $\gamma1$ | 0.70            | 0.48             | +0.22      |
| $\gamma2$ | 0.27            | 0.29             | -0.02      |
| Total in $\gamma$ | 0.97          | 0.77             | +0.20      |

Thylakoids were illuminated in the presence of 5 mM diethiothreitol and the thiol reagent was removed by pelleting the thylakoids. The thylakoids were then exposed to 2 mM N-ethylmaleimide in the light or dark for 2 min. CF$_1$ was isolated from the two thylakoid preparations and denatured with 1\% sodium dodecyl sulfate and heat (100°C, 1 min). $[^3H]$N-ethylmaleimide was added (8 mM, 90,000 cpm/nmol) and after 5 min at room temperature, unreacted maleimide was removed. Aliquots containing 9 pg of protein were subjected to electrophoresis on a 9\% polyacrylamide gel in the presence of sodium dodecyl sulfate. The electrophoresis was carried out for 10 h at 100 V. After staining, the bands were cut out and radioactivity was determined. $\gamma1$ and $\gamma2$ refer to the slower and faster migrating $\gamma$ species, respectively. NEM-dark CF$_1$ and NEM-light CF$_1$ refer to the preparations from thylakoids kept in the dark or illuminated in the presence of N-ethylmaleimide, respectively. Dark-light refers to the H incorporation into NEM-dark CF$_1$, minus that into NEM-light CF$_1$.  

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Effects of N-ethylmaleimide and iodosobenzoate treatment in the dark on light-induced ATPase activity and photophosphorylation

Thylakoids were illuminated in the presence of 5 mM dithiothreitol, washed to remove the thiol reagent, and incubated in the presence or absence of 1 mM N-ethylmaleimide for 2 min in the dark at room temperature. Each suspension was divided in half and 2 mM iodosobenzoate added to one-half. The resulting four thylakoid suspensions were illuminated for 90 s in the presence or absence of 2 mM iodosobenzoate, and phosphorylation with pyocyanine induced ATPase activity, with or without 5 mM dithiothreitol in the light activation mixture, and photophosphorylation with pyocyanine as the mediator of electron flow. The samples for photophosphorylation were illuminated for 90 s in the presence or absence of 5 mM dithiothreitol prior to assay. DTT, dithiothreitol; NEM, N-ethylmaleimide; IBZ, iodosobenzoate.

| Thylakoid treatment | Light-induced Mg\(^{2+}\)-ATPase preillumination | Photophosphorylation preillumination |
|---------------------|--------------------------------|----------------------------------|
| -NEM, -IBZ          | 24 ± 169                        | 534 ± 590                        |
| +NEM, -IBZ          | 297 ± 296                       | 718 ± 756                        |
| -NEM, +IBZ          | 25 ± 179                        | 463 ± 550                        |
| +NEM, +IBZ          | 307 ± 299                       | 650 ± 639                        |

TABLE V
Effects of N-ethylmaleimide treatment in the dark on the inhibition of light-induced Mg\(^{2+}\)-ATPase activity by iodosobenzoate and of photophosphorylation by N-ethylmaleimide

Thylakoids were incubated with or without 2 mM N-ethylmaleimide for 5 min in the dark at room temperature. Unreacted maleimide was removed by washing and both preparations were illuminated for 5 min in the presence of 5 mM dithiothreitol. After removal of the thiol compound by washing, each suspension was divided in half. One-half was incubated with 2 mM N-ethylmaleimide for 5 min in the dark at room temperature and the other half was untreated. The resulting four suspensions were centrifuged and the pellets were resuspended. ATPase activity was assayed after light induction in the presence or absence of 2 mM iodosobenzoate, and phosphorylation was assayed after a 90-s preillumination or dark period in the presence or absence of N-ethylmaleimide, NEM, N-ethylmaleimide; IBZ, iodosobenzoate.

The differences in decay rates. In some experiments, the decay even in well-washed thylakoids was very slow (see, for example Table V). However, o-iodosobenzoate always rapidly inactivates Mg\(^{2+}\)-ATPase activity, whether it is present during illumination (Table V) or in the dark (not shown). CuCl\(_2\) (10 \(\mu\)M) treatment in the dark also rapidly inhibits light-induced Mg\(^{2+}\)-ATPase activity of thylakoids.

Treatment of thylakoids, previously illuminated in the presence of dithiothreitol, with 2 mM N-ethylmaleimide in the dark protects light-induced Mg\(^{2+}\)-ATPase activity from either spontaneous inactivation (Table IV) or iodosobenzoate inhibition (Table V), probably because alkylation of the thiol groups prevents the reformation of the disulfide linkage.

**TABLE VI**
Protection of ATPase activity activated by illumination of intact pea chloroplasts from inactivation by oxidants

Intact pea chloroplasts (0.3 mg of chlorophyll-mL\(^{-1}\)) were illuminated (2 \(\times 10^6\) ergs cm\(^{-2}\) s\(^{-1}\)) in resuspension buffer (50 mM HEPES-NaOH (pH 7.0), 330 mM sorbitol, 25 mM KC\(_1\), 2 mM EDTA, 1 mM Mg\(_{2+}\), and 1 mM Mn\(_{2+}\)) for 5 min at 20°C. They were then lysed by a 5-fold dilution into either water or 2 mM N-ethylmaleimide. After 2 min in the dark at room temperature, the suspensions were centrifuged, and the pellets were washed with a buffered sucrose solution (0.4 M sucrose, 0.02 M Tricine-NaOH (pH 8.0), 0.01 M NaCl), and were resuspended in the buffered sucrose solution. Aliquots of the suspensions (1 mg of chlorophyll-mL\(^{-1}\)) were incubated in the dark for 45 min at 0°C with either no addition, 10 \(\mu\)M CuSO\(_4\), 1 mM iodosobenzoate, or 1 mM K\(_3\)Fe(CN)\(_6\). Aliquots of each suspension were illuminated in the absence of dithiothreitol and ATPase activity was assayed.

**TABLE VII**
Protection of ATPase activity activated by illumination of intact pea chloroplasts from inactivation by oxidants

| Treatment of thylakoids with NEM | Light-induced Mg\(^{2+}\)-ATPase & Photophosphorylation |
|---------------------------------|-----------------|
| None                            | -IBZ +IBZ       |
| After activation                 | 266 ± 149       |
| Before activation                | 190 ± 12        |
| Before and after activation      | 201 ± 157       |

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Fig. 3. Effect of light and dithiothreitol activation on the relationship between phosphorylation rate and ΔpH. Thylakoids (0.25 mg of chlorophyll·ml⁻¹) were illuminated in the presence or absence of 10 mM dithiothreitol for 50 s. Aliquots of the mixtures were diluted 1:2.5 with a pyocyanine-containing phosphorylation or ΔpH reaction mixture. Phosphorylation and ΔpH were determined with a saturating ADP concentration at light intensities that ranged from 0.1 to 2 × 10⁶ ergs·cm⁻²·s⁻¹. Rates are reported as micromoles of P₃ incorporated·h⁻¹·mg⁻¹ chlorophyll. The data points for the dithiothreitol-treated thylakoids correspond in light intensity to the central three control points. DTT, dithiothreitol.

Fig. 4. Effect of the treatment of activated thylakoids with N-ethylmaleimide in the dark on the relationship between phosphorylation rate and ΔpH. Thylakoids were either illuminated or kept in the dark in the presence of 5 mM dithiothreitol. The dithiothreitol was removed by washing. One-half of the light- and dithiothreitol-treated sample was incubated with 2 mM N-ethylmaleimide in the dark for 7 min, whereas no N-ethylmaleimide was added to the other half. Thylakoids treated with dithiothreitol in the dark were also incubated with 2 mM N-ethylmaleimide. The three suspensions were centrifuged and pellets were resuspended in a buffered mannitol solution (0.3 M mannitol, 0.02 M Tricine-NaOH (pH 8), 0.01 M NaCl). The suspensions were centrifuged again, suspended in a small volume of the buffered mannitol solution containing 5% (v/v) dimethyl sulfoxide, and stored at −70 °C. The next day, phosphorylation rates and ΔpH were determined in the same Microfuge tubes with pyocyanine as the mediator of electron flow over a light intensity range of 0.5 to 5 × 10⁶ ergs·cm⁻²·s⁻¹. A saturating ADP concentration (1.0 mM) was used. Rates are expressed as micromoles of P₃ incorporated·h⁻¹·mg⁻¹ chlorophyll. O, thylakoids treated with dithiothreitol in the light, then with N-ethylmaleimide in the dark; ◦, thylakoids treated with dithiothreitol in the dark, then with N-ethylmaleimide in the dark; Δ, thylakoids treated with dithiothreitol in the light, then no N-ethylmaleimide in the dark.

Fig. 5. The relationship between phosphorylation rate and ΔpH in washed pea thylakoids and in freshly lysed chloroplasts. Phosphorylation and ΔpH were measured at several light intensities ranging from 0.1 to 5 × 10⁶ ergs·cm⁻²·s⁻¹ using ferricyanide (1 mM) as the electron acceptor and a saturating ADP concentration (0.5 mM) in either washed pea thylakoids (control) obtained from the upper layer of Percoll gradient or intact pea chloroplasts.

DISCUSSION

Our results suggest that the γ subunit of CF1 in washed thylakoid membranes contains one disulfide bond and two free thiols, similar to soluble CF1 (18, 19, 22). The accessible thiol group reacts readily with alkylating reagents and appears to be unessential for activity, whereas in thylakoids the other thiol reacts with maleimides only when the membranes are energized. Alkylation of this thiol group inhibits photophosphorylation and ATPase activity. As in the case with the soluble enzyme (18, 19), reduction of the disulfide bond of the γ subunit of CF1 in thylakoids activates ATPase activity. This compared to that catalyzed by washed pea thylakoids (Fig. 5). The relatively poor activation at high light intensities may be the result of oxidation of thiol groups to a disulfide by the ferricyanide in the reaction mixture. The internal aqueous volumes and the passive proton permeabilities of the two preparations were the same (not shown). Moreover, the maximal phosphorylation efficiencies (P/e₂ ratios), extrapolated from a plot of observed P/e₂ ratio versus the ratio of internal proton concentration to that of electron flow (38), were the same (1.36). Thus, activation is not caused by a change in the ratio of protons translocated per ATP synthesized.

Dithiothreitol in the assay mixture also activates photophosphorylation at a low ADP concentration (10 μM) (Fig. 6). The effect of dithiothreitol is more pronounced at lower ΔpH values. At this ADP concentration, the phosphorylation rate can approach saturation with respect to ΔpH. We do not observe such saturation at 1.5 mM ADP (Fig. 3), presumably because it would require higher ΔpH values than we obtain with our thylakoid preparations even at saturating light intensities. These data indicate that the reduction of CF1 does not increase the maximal rate of ATP synthesis observed at given ADP and P₃ concentrations and saturating ΔpH, the V₉₀₀₀₉ at saturating ΔpH.

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reduction is enhanced by illumination of thylakoids and is uncoupler sensitive (4, 5, 7). Conformational changes in the γ subunit of membrane-bound CF1, that occur during energization (2, 35) may increase the exposure of the disulfide bond to reduction by dithiothreitol. Although activation of the ATPase activity of soluble or membrane-bound CF1 in the dark is nearly complete within 2 h in the presence of 10 mM dithiothreitol, activation in illuminated thylakoids is maximal within 5 min with only 5 mM dithiothreitol (4).

A subpopulation of CF1 in thylakoids exists that is susceptible to neither reduction nor alkylation of the buried sulfhydryl. This subpopulation is probably unable to undergo energy-dependent conformational changes. No CF1, alkylated at the buried sulfhydryl but remaining oxidized, was observed. However, some CF1 is reduced, but not alkylated at the buried sulfhydryl, probably because reduction can occur at a lower ΔpH than can alkylation. This subpopulation may explain the observation that modification of the buried sulfhydryl in thylakoids with N-ethylmaleimide not only lowers the $V'_{\text{max}}$ for photophosphorylation but also lowers the $K'_{\text{m}}$ for ADP (41). Lowering the light intensity also lowers the $K'_{\text{m}}$ for ADP (42). If this subpopulation of CF1 is on thylakoids that generate a ΔpH sufficient to allow ATP synthesis, but too low to allow the alkylation of the buried sulfhydryl, then the inhibition by N-ethylmaleimide of phosphorylation by those thylakoids able to generate larger ΔpH values would be expected to lower the observed $K'_{\text{m}}$. Alternatively, CF1 modified by N-ethylmaleimide at S3 might retain a reduced catalytic activity having a lower $K'_{\text{m}}$ for ADP. However, preliminary results indicate that the ratio of light-dependent incorporation of N-ethylmaleimide per CF1 is very similar to the fraction of activity inhibited, suggesting that each alkylated CF1 is completely inhibited.8

Reduction is sufficient to activate the ATPase activity of the soluble enzyme (19) and is associated with changes in the conformation of the γ subunit (19, 43). In contrast, reduction is not sufficient for the activation of the ATPase activity of thylakoids. Even when the enzyme is reduced, a period of illumination is required to induce ATPase activity (7). This induction is also sensitive to uncouplers. The requirement for energy to induce the ATPase activity in reduced membranes may be explained, at least in part, by the observation (8, 9) that ADP dissociates from the enzyme in the light and reassociates in the dark. The binding of ADP to thylakoids after a period of illumination has been correlated to the decay of ATPase activity in the dark (8, 9).

In the presence of high concentrations of ADP and P_i, reduced CF1 in thylakoids catalyzes a higher rate of ATP synthesis at a given ΔpH than oxidized CF1. Similarly, in the presence of a low concentration of ADP, reduced CF1 catalyzes a higher rate of ATP synthesis at a given low ΔpH than oxidized CF1. However, reduction of CF1 does not increase the $V'_{\text{max}}$ at saturating ΔpH. This suggests that reduction of CF1 activates ATP synthesis by promoting the interaction of internal protons with the coupling factor complex or the rate of some other external step which is dependent on internal and probably external proton concentrations. Such an elementary step would be rate-limiting only at relatively low ΔpH values. This conclusion is consistent with the results of Vallejos et al. (14) on the activation of CF1 by illumination of leaves: the resulting stimulation of photophosphorylation at high light intensities was found to be greater at high ADP concentrations than at low ADP concentrations, probably because ΔpH is a rate-limiting factor at high light intensities and high ADP concentrations, but not rate limiting at high light intensities and low ADP concentrations (compare Figs. 3 and 4).

The effects of ΔpH and Δ$\gamma$ on the rates of ATP synthesis and hydrolysis catalyzed by thylakoids have been described in terms of an energy-dependent equilibrium between active and inactive states of CF1 (44, 45). According to this view, at a given ΔpH, the fraction of reduced CF1 in the active conformation is greater than that of oxidized CF1. Within this framework, one can consider two limiting mechanisms by which internal protons increase the observed rate of ATP synthesis: they may interact with the coupling factor complex as substrates, three of which are translocated per ATP synthesized (38, 46, 47), or as allosteric activators, which are not translocated during turnover. Whatever the roles of protons may be, it is clear that the oxidation state of the γ subunit of CF1 plays a central role in regulation of photophosphorylation.

It is interesting to contrast the regulation of the chloroplast coupling factor to that of the Escherichia coli and mitochondrial coupling factors. While the chloroplast 5-subunit coupling factor is isolated as a latent ATPase, those of E. coli and mitochondria are isolated as active ATPases (48). In addition, the mitochondrial coupling factor is regulated by a soluble inhibitor protein (49). Presumably, these differences reflect the different physiological roles played by these coupling factors. Even in the absence of oxidative substrates, the mitochondrial and E. coli coupling factors remain active and catalyze ATP hydrolysis, which maintains the electrochemical proton gradient required for other processes including the transport of nutrients (50, 51). On the other hand, the inactivation of the chloroplast coupling factor in the dark may prevent wasteful ATP hydrolysis.

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