Sulfhydryl Reagents and Energy-Linked Reactions in Monocot Thylakoids

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ABSTRACT

Monofunctional maleimides have been used to covalently modify the coupling factor protein of monocot thylakoid membranes. As with dicot thylakoids, incubation of the monocot thylakoids with maleimides in the light but not in the dark results in inhibition of both ATP synthesis and hydrolysis. In the dark, sites on the \( \gamma \) and \( \epsilon \) subunits of maize Zea mays coupling factor 1 are modified after incubation of maize mesophyll thylakoids with the fluorescent maleimide \( N \)-((anilinonaphthyl-4)-maleimide, APBM, dithiobisnitropyridine; \( \text{DTNP} \), and anilinonaphthyl-4)maleimide, \( \text{APN} \). A light accessible site localized solely to the \( \gamma \) subunit has also been demonstrated. In contrast to the case with dicot thylakoids (spinach \( \text{Spinacia oleracea} \) and pea \( \text{Pisum sativum} \)) treatment of monocot thylakoids (maize, barley \( \text{Hordeum vulgare} \), crabgrass \( \text{Digitaria sanguinalis} \)) with bifunctional maleimides or thiol oxidants in the light does not affect proton coupling, i.e., the bifunctional reagents act more like energy transfer inhibitors. The lack of functional uncoupling could be due either to a failure of the reagents to cross-link key sulfhydryl residues in the \( \gamma \) subunit or to the continued ability of the \( \gamma \) subunit to gate proton movements through the chloroplast coupling factor complex even though its conformation has been altered by sulfhydryl reagents.

The \( \gamma \) subunit of the spinach proton-ATPase contains one disulfide bridge and two free sulfhydryls (9). One of the free sulfhydryls (S4 in McCarty’s terminology (15)) is readily accessible to modification by maleimides in the dark. Modification of S4 has no apparent effect on energy transduction mediated by the coupling factor (CF;\(^1\)). The second free sulfhydryl (S3) is not readily accessible, but becomes available when the thylakoid membrane is energized. Modification of S3 with maleimides inhibits both ATP synthesis and hydrolysis without affecting proton leakage (10, 12). With spinach thylakoids inhibition of CF; catalytic activities by monofunctional maleimides is energy dependent (10). Whereas with \( \text{Dunaliella} \) and \( \text{Spirulina} \) membranes maleimide inhibition of CF; does not require a light-induced protonotive force (6, 20). When S3 (Cys-89) and S4 (Cys-322) of spinach \( \gamma \) are cross-linked by bifunctional maleimides or by reagents that oxidize vicinal dithiols, e.g., \( \text{DTNP} \), the thylakoids are rendered proton leaky (16).

Of interest to us has been the question of how general these phenomena are with regard to the gamma subunits of coupling factors other than spinach CF;\(^1\). In the present work we have extended the study of the interaction of sulfhydryl reagents with the proton ATPase to the mesophyll thylakoids of monocots.

MATERIALS AND METHODS

Zea mays (Pioneer hybrid #3747 or FR9cms × FR37), \( \text{Hordeum vulgare} \) (var Barsoy), and \( \text{Pisum sativum} \) (Progress #9) were grown in a greenhouse with a 15-h photoperiod maintained with supplemental low pressure sodium vapor lamps and incandescent lamps. \( \text{Digitaria sanguinalis} \) plants were grown in the summer in a greenhouse under natural illumination. Spinach was obtained from local markets. Mesophyll thylakoids were isolated from 50 to 60 g of leaf material chopped into 0.5 to 1.0 cm segments. The segments were homogenized with a Polytron PT-35 at a setting of “6” for 5 s in 300 mL of a basic medium containing 400 mm sorbitol, 50 mm Tricine-Na\(^+\) (pH 7.8), 10 mm NaCl, and 2 mm MgCl\(_2\). For spinach, pea, and barley the grinding medium also contained 0.05% BSA and 0.1% ascorbate. For maize and crabgrass, the grinding medium contained 0.2% BSA, 0.2% ascorbate and 2 g of Polyclar AT. The homogenate was filtered through a combination of nylon bolting cloth and Miracloth and centrifuged for 5 min at 3000g to collect the thylakoids. The thylakoids were washed once (3000g) for 5 min with basic grinding medium and resuspended in the same medium. Chl was estimated spectrophotometrically in 80% acetone extracts (1).

Oxygen evolution was assayed polarographically in rate-saturating orange light (2). The assay mixture contained 100 mm sorbitol, 25 mm Tricine-Na\(^+\) (pH 8.1), 25 mm NaCl, 5 mm MgCl\(_2\), 0.1 mm methyl viologen, 0.5 mm NaN\(_3\), 0.1 mm ATP, and thylakoids equivalent to 15 to 25 \( \mu \)g Chl/ml. Ca\(^{2+}\)-ATPase activity associated with soluble CF; was assayed as described previously (13). Activation of thylakoids for subsequent dark assay of Mg\(^{2+}\)-ATPase activity was performed as follows. Thylakoids equivalent to 100 \( \mu \)g Chl/ml were illuminated (2 \( \times \)10\(^6\) ergs/cm\(^2\)-s of white light) for 5 min (25°C) in a medium containing 50 mm NaCl, 50 mm Tricine-Na\(^+\) (pH 8.1), 5 mm MgCl\(_2\), 0.05 mm PMS and 10 mm DTT. SO\(_4\)\(^2-\)-dependent Mg\(^{2+}\)-ATPase assays contained 50 mm Tricine-Na\(^+\) (pH 7.8), 4 mm ATP, 2 mm MgCl\(_2\), 1 mm N\(_2\)H\(_2\), 50 mm NaSO\(_4\), and light- and DTT-activated thylakoids.

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\(^{3}\) Abbreviations: CF; chloroplast coupling factor one; ANM, \( N \)-((anilinonaphthyl-4)-maleimide; APBM, azophenylbismaleimide; DTNP, dithiobisnitropyridine; MeNH\(_2\), methylamine; NEM, N-ethylmaleimide; NPM, N-phenylmaleimide; OPBM, \( \alpha \)-phenylethylmaleimide; PMS, phenazine methosulfate.
equivalent to 10 µg Chl (8). The assays were performed at 37°C for 10 min. ATP synthesis was assayed as described elsewhere (3, 4). Pi was estimated colorimetrically (21).

Maize CF₁ was isolated from maize mesophyll thylakoids employing the chloroform release method of Younis et al. (23). The protein was judged to be 90% pure by SDS-polyacrylamide electrophoresis. ANM labeling of soluble or membrane-bound maize CF₁ was carried out as described by McCarty and co-workers (17, 18). Fluorescence measurements were made with a Perkin-Elmer number 650-10S spectrophotofluorometer. ANM fluorescence was excited at 355 nm.

APBM, NPM, and OPBM were gifts from Dr. R. E. McCarty. ANM was obtained from Serva. DTNP and NEM were obtained from Sigma. All other chemicals were of the highest grade commercially available.

RESULTS

Maize mesophyll thylakoids are similar to spinach thylakoids with regard to inhibition of photophosphorylation by monofunctional maleimides. Inhibition by NPM is only observed in energized membranes with an Iₐₐ value for PMS-catalyzed phosphorylation of approximately 20 µM (Fig. 1). Both the SO₄²⁻-dependent Mg²⁺-ATPase and ATP synthase activities of maize mesophyll thylakoids are inhibited to a similar extent by the monofunctional maleimides NPM and ANM (Table I). Treating spinach thylakoids with bifunctional maleimides (OPBM, APBM) or the thiol oxidant DTNP renders the thylakoids proton leaky (which is indicated by an increase in the rate of non-phosphorylating electron flow) (14, 16). When spinach thylakoids were treated, under appropriate conditions in the light with OPBM there was approximately a threefold increase in the rate of basal electron transport (Fig. 2). In contrast, treatment of maize mesophyll thylakoids with OPBM was without effect. Table II shows a summary of a number of experiments in which the effects of APBM (cross-linking distance = 19Å), OPBM (cross-linking distance = 6Å) and DTNP on non-phosphorylating electron flow were examined in both dicot and monocot thylakoids. Only in the case of dicot thylakoids were stimulatory effects on basal electron transfer observed.

To determine whether the bifunctional maleimides were capable of cross-linking sulfhydryl residues in the γ subunit of maize CF₁, we compared the effect of OPBM (a bifunctional reagent) and NPM (a monofunctional reagent) directly on ATP synthase or indirectly using the assay conditions of Weiss and McCarty (22). Their procedure involves incubating thylakoids in the dark with the bifunctional reagent (binding to S4), removing the reagent by washing, energizing the washed thylakoids (cross-linking of S3 and S4), and then assaying ATP synthesis. In the direct assays NPM and OPBM

| Table I. Effect of Monofunctional Maleimides on ATP Synthesis and Hydrolysis |
|--------------------------|------------------|------------------|
| Assay                    | Control          | ATP synthase     |
|                          | +50 µM NPM       | +30 µM ANM       |
| SO₄⁻-Mg²⁺-ATPase (µmol/mg Chl/h) | 215 (0.21)       | 135 (0.21)       |
| ATP synthesis            | 738              | 49 (0.16)        |

* The numbers in parentheses indicate percent of the control.

**Figure 1. NPM inhibition of PMS-catalyzed photophosphorylation.** Maize mesophyll thylakoids equivalent to 200 µg Chl (2 ml) were either illuminated with white light (2 × 10⁶ ergs/cm²/s) for 2 min at room temperature or incubated in the dark in the presence or absence of varying concentrations of NPM in the following medium: 50 mM NaCl, 50 mM Tricine-Na⁺ (pH 8.1), 5 mM MgCl₂ and 0.05 mM PMS. After the pretreatment, DTTP was added to a final concentration of 0.2 mM. The treated samples (20 µg Chl) were then assayed for PMS-dependent cyclic photophosphorylation. The control rates of ATP synthesis were 495 and 423 µmol/mg Chl/h for samples incubated in the dark and in the light, respectively.

**Figure 2. Effect of OPBM on non-phosphorylating electron flow.** Maleimide treatment of maize mesophyll and spinach thylakoids in the light was performed as described in the legend to Figure 1. The illuminated samples (13-15 µg Chl/ml) were then employed in electron transport assays with methyl viologen as acceptor.
inhibited ATP synthesis 40 to 60% with both spinach and maize mesophyll thylakoids (Table III). In the cross-linking experiments NPM did not inhibit with either maize or spinach thylakoids. With OPBM inhibition was only observed with spinach thylakoids. The failure of the bifunctional reagent to cross-link and functionally uncouple maize mesophyll thylakoids could be due to a number of factors: (a) the reagent does not bind to S4 in the dark, (b) the γ subunit is lacking S3, (c) the environment around S3 is quite different in the spinach and maize mesophyll γ subunits, and (d) cross-linking S3 and S4 in monocot γ subunits does not render the ATPase complex proton leaky.

To gain more information concerning the distribution of sulfhydryl residues in the maize γ subunit, we employed the fluorescent maleimide ANM to tag the cysteines of maize mesophyll CF1, both on and off the membrane. The results we obtained with the soluble enzyme are similar to those reported by Patrie and Miles (19) for maize CF1, and Nalin and Mccarty (17) for spinach CF1. Very little ANM labeling is observed with the latent protein. Activation of Ca2+-ATPase activity after treatment with 50 mM DTT for 4 h selectively increases the labeling of the gamma subunit (Fig. 3). After blocking the accessible sulfhydryls with NEM, one observes after DTT treatment an increase in the ANM labeling of the γ subunit and a change in the mobility of γ (Fig. 4). In agreement with the observations of Patrie and Miles little if any labeling of the ε subunit is observed with the soluble protein.

When CF1 was isolated from maize mesophyll thylakoids that had been incubated in the dark with 30 mM ANM both the γ and ε subunits were labeled (Fig. 5). Pretreatment of the membranes with NEM completely eliminated the ANM labeling. When the ANM labeling was performed under energized conditions both γ and ε were labeled. Pretreatment of the membranes with NEM eliminated the labeling of the ε subunit but not labeling of the γ subunit (Fig. 6).

When ANM is protein bound, there is a blue shift in the fluorescence emission maximum (7). Spinach CF1, modified with ANM (NEM pretreated) exhibits a fluorescence maximum at 430 nm (18). When maize mesophyll CF1 was labeled

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**Table II. Effect of Sulphydryl Reagents on Electron Transport in Dicot and Monocot Thylakoids**

<table>
<thead>
<tr>
<th>Thylakoid Preparation</th>
<th>Rate of eT + Addition/Rate of eT − Addition</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>10 μM OPBM</td>
</tr>
<tr>
<td>Spinach</td>
<td>2.91</td>
</tr>
<tr>
<td>Pea</td>
<td>2.57</td>
</tr>
<tr>
<td>Maize</td>
<td>0.90</td>
</tr>
<tr>
<td>Crabgrass</td>
<td>1.28</td>
</tr>
<tr>
<td>Barley</td>
<td>1.12</td>
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</tbody>
</table>

**Table III. Effect of OPBM on ATP Synthesis in Spinach and Maize Mesophyll Thylakoids**

Thylakoids (100 μg Chl/ml) were incubated in the dark for 5 min at room temperature with either OPBM, NPM or in the absence of maleimides. The thylakoids were then washed and resuspended as described in the legend to Figure 2. These thylakoids were either preilluminated or kept in the dark for 2 min prior to the assay of photophosphorylation. Direct inhibition assays were performed as described in the legend to Figure 1.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Dark/Light Inhibition</th>
<th>Direct Light Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/mg Chl/h</td>
<td>%</td>
</tr>
<tr>
<td>A (Maize)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>168.9</td>
<td></td>
</tr>
<tr>
<td>50 μM NPM</td>
<td>164.3</td>
<td>52</td>
</tr>
<tr>
<td>10 μM OPBM</td>
<td>176.9</td>
<td>62</td>
</tr>
<tr>
<td>B (Spinach)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>607.5</td>
<td></td>
</tr>
<tr>
<td>50 μM NPM</td>
<td>591.4</td>
<td>43</td>
</tr>
<tr>
<td>10 μM OPBM</td>
<td>475.2</td>
<td>45</td>
</tr>
</tbody>
</table>
absence (15 mM) pretreated with 2 mM NEM for 30 min at room temperature in the Tris-EDTA-ATP medium described in the legend to Figure 3. DTT (15 mM) was added and the protein was subjected to two successive G-50 spin columns. ANM labeling of native, latent and denatured (1% SDS), latent CF₁ was carried out for 5 min at room temperature. ANM labeling was also carried out with NEM-treated CF₁ in the presence of 1% SDS, and with CF₁ that had been NEM-treated and reduced with 15 mM DTT for 30 min in the presence of 1% SDS. The procedures employed after ANM labeling were similar to those described in the legend to Figure 3. Coomassie staining: lane A, SDS + NEM + DTT. B, SDS + NEM. ANM fluorescence: lane C, SDS + NEM + DTT. D, SDS + NEM. E, SDS + DTT. F, --SDS.

under similar conditions, one observed a blue shift in the ANM emission maximum to approximately 427 nm (data not shown).

**DISCUSSION**

From our ANM labeling experiments with both soluble and membrane-bound maize mesophyll CF₁, it appears that the γ subunit is the major site of interaction with maleimides. Our labeling experiments with the soluble, activated protein are consistent with the presence of a disulfide bridge in maize gamma. Labeling experiments with the membrane-bound protein indicate a dark binding site for maleimides as well as a site on γ that is only accessible in the light. The environment around the light site appears to be similar in both the spinach and maize γ subunits. In spinach, S3 (Cys-89) is surrounded by a number of glycine residues and is in a quite hydrophobic environment (11). The observation that bifunctional reagents, e.g. OPBM, act more like monofunctional reagents with

**Figure 4.** ANM labeling of soluble maize CF₁ in the presence and absence of NEM. Desalted maize CF₁ equivalent to 100 μg protein was pretreated with 2 mM NEM for 30 min at room temperature in the Tris-EDTA-ATP medium described in the legend to Figure 3. DTT (15 mM) was added and the protein was subjected to two successive G-50 spin columns. ANM labeling of native, latent and denatured (1% SDS), latent CF₁ was carried out for 5 min at room temperature. ANM labeling was also carried out with NEM-treated CF₁ in the presence of 1% SDS, and with CF₁ that had been NEM-treated and reduced with 15 mM DTT for 30 min in the presence of 1% SDS. The procedures employed after ANM labeling were similar to those described in the legend to Figure 3. Coomassie staining: lane A, SDS + NEM + DTT. B, SDS + NEM. ANM fluorescence: lane C, SDS + NEM + DTT. D, SDS + NEM. E, SDS + DTT. F, --SDS.

**Figure 5.** ANM labeling of membrane-bound CF₁ in the dark. Maize mesophyll thylakoids equivalent to 10 mg Chl were incubated (0.1 mg Chl/mL) in the dark in a medium containing 50 mM NaCl, 50 mM Tricine-Na⁺ (pH 8.0), 2.5 mM MgCl₂ and 0.03 mM ANM for 5 min at room temperature with stirring. After the incubation period, DTT was added to a final concentration of 0.5 mM. The thylakoids were collected by centrifugation (5000g for 10 min) and resuspended in a medium containing 10 mM Tricine-Na⁺ (pH 8.0), 5 mM NaCl. The thylakoids were allowed to swell on ice for 10 min. They were pelleted by centrifugation (10,000g for 15 min) and resuspended in a medium containing 250 mM sucrose, 20 mM Tricine-Na⁺ (pH 7.6), 1 mM EDTA, 1 mM ATP and 5 mM DTT. CF₁ was then isolated by the chloroform release method and the protein was stored as a suspension in 50% (NH₄)₂SO₄ at 4°C. Prior to electrophoresis, CF₁ was desalted as described in the legend to Figure 3. NEM pretreatment of thylakoid membranes was carried out by incubating thylakoids (100 μg Chl/mL) with 2 mM NEM in the medium described above for 15 min at room
monocot thylakoids could be explained then either by assuming that these reagents do not cross link the dark and light sites or that a proton ATPase complex with a cross-linked γ subunit does not lose its proton gating function.

With algal and cyanobacterial coupling factors the sulfhydryl residue critical for catalytic activity appears to be accessible to covalent modification by maleimides in the dark under nonenergized conditions. Monocot γ subunits appear to be similar to dicot γ subunits in that covalent modification occurs only under energized conditions. In both spinach and cyanobacteria the reactive cysteine appears to be at residue 89 or 90 in the primary structure of the gamma subunit (5, 11). Primary sequence data for the maize γ subunit is not presently available.

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LITERATURE CITED


Figure 6. ANM labeling of membrane-bound CF1 in the light. Mesophyll thylakoids were incubated in the light (>2 × 106 ergs/cm²/s of white light) for 2 min at room temperature in a medium containing 50 mM NaCl, 50 mM Tricine-Na+ (pH 8.1), 2.5 mM MgCl2, 0.05 mM PMS and 0.03 mM ANM. After the illumination period, DTT was added to a final concentration of 0.5 mM. Release of CF1, NEM pretreatment and electrophoresis procedures were as described in the legends to Figures 3 and 4. Lane A, +NEM; B, −NEM.

temperature in the dark. DTT was then added to a final concentration of 7.5 mM. The thylakoids were collected by centrifugation (6000g for 5 min), washed once (6000g for 5 min) with basic grinding medium plus 1 mg/mL BSA and resuspended in a small volume of the basic grinding medium minus BSA. Lane A, +NEM; B, −NEM.


