The Distance Between Thiol Groups in the γ Subunit of Coupling Factor 1 Influences the Proton Permeability of Thylakoid Membranes

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Abstract

Spinach chloroplast thylakoids treated in the light with bifunctional malei-mides were previously shown to be uncoupled. The increase in proton permea-bility by these reagents is caused by the cross-linking of an accessible group on the γ subunit of coupling factor 1 (CF_1) to a group that becomes exposed to reaction with maleimides only when the thylakoids are energized. In this study, several bifunctional maleimides, including *o*-, *m*-, and *p*-phenylenebismaleim-ides, 2,3- and 1,5-naphthalenebismaleimides, and azophenylbismaleimide, were tested for their ability to form cross-links and to uncouple photophosphor-ylation. These reagents form cross-links from about 6 to 19 Å. Each reagent was found to form cross-links in the light and to inhibit photophosphorylation. However, the effectiveness of these compounds as uncouplers decreased as the distance between the cross-linked groups increased, indicating that the distance between two groups on the γ subunit of CF_1 can regulate proton flux through the membrane. Monofunctional maleimides cause a light-dependent energy transfer type of inhibition of photophosphorylation. Although this inhibition was correlated to the reaction of the maleimide with a group on the γ subunit that is exposed only in energized thylakoids, the accessible group on this subunit was also modified by the reagent. However, we show here that the accessible group plays no role in the inhibition of photophosphorylation. This group may be blocked by incubating thylakoids in the dark with methyl methanethiolsul-fonate. The light-dependent inhibition of photophosphorylation by *N*-ethylmal-eimide was unaffected by this treatment or by the subsequent removal of the methanethiol moiety from the accessible group.

Key Words: Chloroplast coupling factor 1; photophosphorylation; protein cross-linking; conformational change; sulfhydryl reagents.

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Introduction

The γ subunit of coupling factor 1 $(CF_1)^2$ from spinach chloroplast thylakoids contains at least two groups that react with maleimides (McCarty and Fagan, 1973). One group is accessible in the dark and a second is exposed only in the light (McCarty and Fagan, 1973). Modification of the accessible group does not cause inhibition, but modification of the group exposed only in the light by either N-ethylmaleimide or o-phenylenebismaleimide (OPBM) inhibits photophosphorylation (McCarty and Fagan, 1973; Weiss and McCarty, 1977). The manner in which monofunctional maleimides inhibit photophosphorylation differs from that of bifunctional maleimides like OPBM. N-Ethylmaleimide treatment inhibits photophosphorylation but has no effect on light-induced proton uptake (McCarty et al., 1972), the pH differential across the thylakoid membrane (Portis et al., 1975), or nonphosphorylating electron flow (McCarty et al., 1972). In contrast to N-ethylmaleimide, the bifunctional maleimides OPBM (Weiss and McCarty, 1977) and dithiobisethylmaleimide (Moroney and McCarty, 1979) act at least in part as uncouplers. This uncoupling is due to a cross-linking of two groups within the γ subunit of CF_1. Experiments with o-iodosobenzoate and dithiobisnitropyridine (Moroney et al., 1980) indicate that these groups are probably sulfhydryls and that nearby thiols are oxidized to a disulfide. This oxidation also causes uncoupling. These results indicate that the sulfhydryls are close together in illuminated thylakoids. Bifunctional reagents that have longer distances between the maleimides were therefore tested to see whether these compounds would also cross-link within the γ subunit of CF_1. Although cross-linking by all bifunctional maleimides tested occurred, uncoupling was evident only with those reagents that spanned relatively short distances. In general, the longer the span, the less effective was a reagent as a light-dependent uncoupler of photophosphorylation.

Since both the accessible group and that exposed in the light are labeled with N-ethylmaleimide, it was unclear whether both had to be labeled for inhibition of photophosphorylation to occur or whether modification of only the site exposed in the light was sufficient. By reversibly blocking the accessible group with methyl methanethiolsulfonate we can modify only the site exposed in the light with N-ethylmaleimide and show that labeling of this group alone is sufficient for inhibition.

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[2]Abbreviations used: CF_1, coupling factor 1 from spinach chloroplast thylakoids; Tricine, N-Tris(hydroxymethyl)methylglycine; OPBM, o-phenylenebismaleimide; MMPM, m-phenylenebismaleimide; PPBM, p-phenylenebismaleimide; MMTS, methyl methanethiolsulfonate; APBM, azophenylbismaleimide; PAPM, N-(p-phenylazophenyl)maleimide.

Materials and Methods

Spinach chloroplast thylakoids (McCarty and Racker, 1967) were treated with the sulfhydryl reagents using pyocyanine as the mediator of electron flow (Moroney et al., 1980). Briefly, thylakoids equivalent to 100 μg of chlorophyll/ml were incubated in white light (2×10^6 erg/cm^2-s) or in the dark for the indicated times in a mixture containing 50 mM Tricine/NaOH (pH 8.0), 50 mM NaCl, 5 mM MgCl$_2$, 0.02 mM pyocyanine, and sulfhydryl reagent at the concentrations indicated in the figure and table legends. In some experiments the treated thylakoids were collected by centrifugation at $3000 \times g$ for 10 min and washed in a buffered sucrose solution containing 0.4 M sucrose, 0.02 M Tricine/NaOH (pH 8.0), 0.01 M NaCl, and 0.1% bovine serum albumin.

Nonphosphorylating electron flow assay mixtures (1.0 ml) contained thylakoids equivalent to 50 μg of chlorophyll, 50 mM Tricine/NaOH (pH 8.0), 50 mM NaCl, 5 mM MgCl$_2$, 1 mM K$_3$Fe(CN)$_6$, and ATP as indicated. If photophosphorylation was to be assayed, 3 mM ADP and 2 mM potassium phosphate buffer (pH 8.0) containing 10^6 cpm of ^{32}P$_i$ were added and the ATP omitted. After illumination for 1.5 to 2 min at room temperature, trichloro-acetic acid was added to 2%. ^{32}P esterification was determined (Avron, 1960) in aliquots of deproteinized mixtures. In some experiments ^{32}P$_i$ was omitted and the amount of phosphate remaining determined spectrophotometrically (Taussky and Shorr, 1953). Ferricyanide (Jagendorf and Smith, 1962) and chlorophyll (Arnon, 1949) were also determined spectrophotometrically. Transmembrane pH gradients (ΔpH) were estimated from hexylamine distri-butions. [^{14}C]Hexylamine uptake was estimated by silicone fluid centrifuga-tion as described previously (Portis et al., 1975) except that the [^{3}H]sorbitol was omitted and the entire glycerol–trichloroacetic acid layer was taken for determination of radioactivity.

Methyl methanethiolsulfonate was synthesized by the method of Smith et al. (1975). Azophenyl-N-N'-bismaleimide was synthesized by a modifica-tion of the method of Fasold et al. (1963). 4,4'-Azodianiline (0.5 g, 2.36 nmol) in 15 ml of dimethyl sulfoxide was added dropwise to a vigorously stirred solution of maleic anhydride (1.25 g, 12.8 mmol) in 2.5 ml of the same solvent. A drying tube containing anhydrous CaCl$_2$ was attached and the mixture stirred for an additional 9 hr. The cloudy solution was then poured over ice water and the resulting orange precipitate dried and washed with diethyl ether to remove residual 4,4'-azodianiline and maleic anhydride. The product was used directly for sodium acetate-catalyzed ring closure in acetic anhydride (Fasold et al., 1963). The crude bismaleimide thus obtained was recrystallized once from dioxane–ethanol (1:1) and then twice from dioxane. A thorough

washing with diethyl ether was found to facilitate removal of dioxane from the purified product (approximately 20% total yield). TLC on silica gel plates (dioxane–0.8 N NH_3, 5:1) showed a single component, R_f = 0.74. The proton NMR of the compound in $CDCl_3$ was consistent with the structure of azophenyl-N,N'-bismaleimide showing peaks at δ 6.88 (4H, s), 7.61 (4H, d, J = 1.9), and 7.99 (4H, d, J = 2.2). 2, 3- and 1,5-naphthalenebismaleimide were synthesized from the corresponding naphthylamines, essentially as described for the synthesis of o-phenylenebismaleimide (Weiss and McCarty, 1977). N-(p-Phenylazophenyl)maleimide was obtained from Eastman, and OPBM, from Sigma. N-Phenylmaleimide and PPBM were from Aldrich. MPBM was purchased in crude form from K. and K. and was crystallized from acetone/water mixtures. The purity of the naphthalene- and phenylene-bismaleimides, as determined by their cysteine reactivity (Moroney and McCarty, 1979) was greater than 95%.

Results

Some of the bifunctional maleimides used and the distances between the maleimides are shown in Fig. 1. All of these compounds inhibited photophosphorylation only if the thylakoids were illuminated in the presence of the inhibitor prior to assay. This inhibition was prevented if adenine nucleotides were present during the illumination as reported previously for N-ethylmaleimide (McCarty et al., 1972) and OPBM (Weiss and McCarty, 1977). In addition, all of the bifunctional maleimides inhibited photophosphorylation at concentrations of about 5 μM whereas the analogous monofunctional compounds, phenylmaleimide and naphthalenemaleimide, required concentrations 10-fold higher to achieve the same inhibition. These results provide indirect evidence that these compounds inhibit photophosphorylation by forming cross-links within the γ subunit of CF_1 as is the case for OPBM (Weiss and McCarty, 1977).

Further evidence that the longer phenylenemaleimides also form cross-links is that all three of the bifunctional phenylenemaleimides stimulated basal electron flow at low concentrations, while N-phenylmaleimide has no effect (Fig. 2). At higher concentrations this stimulation is less dramatic with all of the bifunctional compounds. This is probably because at high concentrations these bifunctional reagents begin to compete effectively with the reagent already bound to the site accessible in the dark so that one molecule reacts with the exposed site while a second molecule reacts with the site exposed only in the light. This prevents cross-linking and causes an energy transfer type of inhibition (Moroney et al., 1980). Additional evidence of uncoupling by these reagents is shown in Table I. The treatment of thylakoids with either
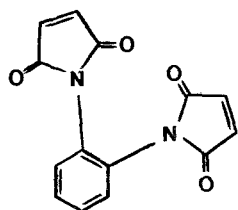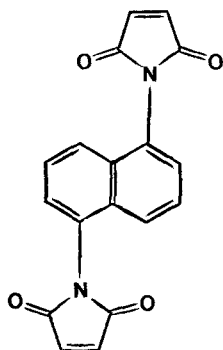
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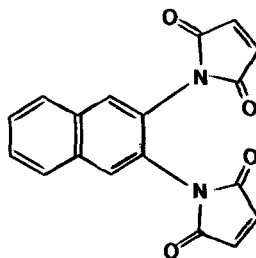(OPBM)
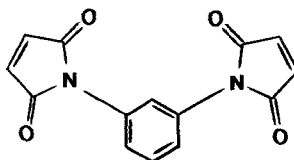
m-phenylenebismaleimide
(MPBM)
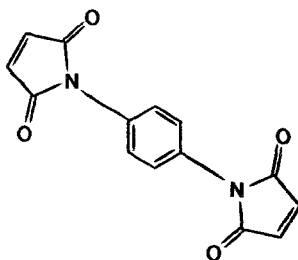
p-phenylenebismaleimide
(PPBM)

1,5-naphthalenebismaleimide   2,3-naphthalenebismaleimide

Fig. 1. Bifunctional maleimides used for cross-linking CF$_1$. The center-to-center distance between the cross-linked thiols was estimated from consideration of CPK space filling models. The compounds used and the estimated cross-link distances are: o-phenylenebismaleimide, (5–6 Å); m-phenylenebismaleimide, (8.5–12 Å); p-phenylenebismaleimide, (12–13 Å); 1,5-naphthalenebismaleimide, (13–15 Å); 2,3-naphthalenebismaleimide, (5–6 Å).
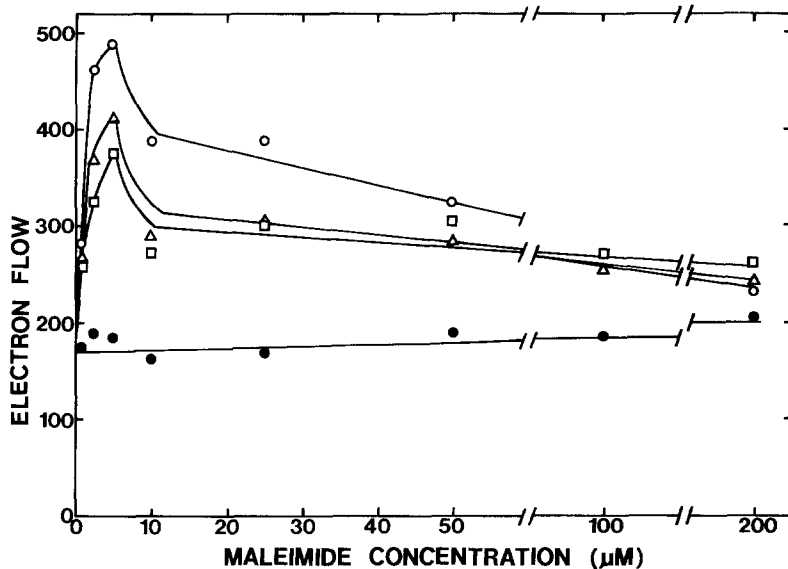
Fig. 2. Stimulation of basal electron transport by bifunctional phenylenemaleimides. Thylakoids (100 μg chlorophyll/ml) were illuminated in the presence of 20 μM pyocyanine and the indicated maleimide for 60 s. The thylakoids were then diluted 1:1 with a solution containing 50 mM Tricine/NaOH (pH 8.0), 50 mM NaCl, 5 mM MgCl$_2$, 2 mM K$_3$Fe(CN)$_6$, and 200 μM ATP. Ferrocyanide production was then measured. The maleimides used were p-phenylenebismaleimide ($\square$), m-phenylenebismaleimide ($\triangle$), o-phenylenebismaleimide ($\circ$), and N-phenylmaleimide ($\bullet$). Rates are expressed as μmol ferricyanide reduced/mg chl-hr.

Table I. Effect of Various Monofunctional and Bifunctional Maleimides on Phosphorylating Electron Flow and P/e_2 Ratios[a]

Thylakoid treatment	Preillumination time	e^- flow (μeq/mg chl-hr)	Phosphorylation (μmol P$_i$/mg chl-hr)	P/e_2
Control	20 s	233	94	0.81
2 mM NEM	30 s	181	47	0.53
20 μM Pyrene maleimide	40 s	206	50	0.49
5 μM OPBM	20 s	281	41	0.23
10 μM DTEM	40 s	279	49	0.35
9 μM 1,5-NAPM	20 s	260	46	0.36

[a]Thylakoids were treated with the indicated maleimides for various times in the light. The illumination times shown were chosen so each maleimide inhibited photophosphorylation by about 50%. K$_3$Fe(CN)$_6$ (1 mM) was the acceptor of electron flow for the assay of P/e_2 and 20 μM pyocyanine was present during the preillumination. 1,5-NAPM stands for 1,5-naphthalene-bismaleimide and DTEM for dithiobisethylmaleimide.

N-ethylmaleimide or pyrene maleimide in the light prior to assay lowered the rate of phosphorylating electron flow while all three bifunctional maleimides tested stimulated electron flow. Thus, even when the inhibitor concentrations and illumination times are adjusted so that the inhibition of photophosphorylation is about 50% in all cases, all three bifunctional reagents stimulate phosphorylating electron flow while the two monofunctional reagents tested inhibit electron flow. Bifunctional maleimides, therefore, have a more pronounced effect on the P/e_2 ratio than monofunctional ones. To determine the optimal distance for cross-linking the two groups, three isomers of phenylenebismaleimide were compared for their ability to uncouple. While all three maleimides increased electron transport, o-phenylenebismaleimide stimulated basal electron flow to a greater extent than m-phenylenebismaleimide (MPBM) and p-phenylenebismaleimide (PPBM) (Fig. 2). All three compounds significantly lowered the transmembrane pH gradient (ΔpH) (Table II), while the monofunctional maleimide has no effect. These changes in ΔpH are consistent with the observation (Portis *et al.,* 1975) that relatively small decreases in ΔpH are associated with large increases in the rate of nonphosphorylating electron flow. The effectiveness of these maleimides as uncouplers, as detected by both the stimulation of basal electron flow and decrease in ΔpH, was OPBM > MPBM > PPBM. Moreover, naphthalene-1,5-bismaleimide was a less effective uncoupler than naphthalene-2,3-bismaleimide. Both of these compounds stimulated basal electron flow, and this effect was blocked by pretreating the chloroplasts with N-ethylmaleimide in the dark. Pretreatment of the thylakoids with N-ethylmaleimide blocks the accessible sulfhydryl which is one of the groups cross-linked by the bifunctional reagents. This prevents cross-linking and therefore uncoupling (Weiss and McCarty, 1977; Moroney and McCarty, 1979). The stimulation of electron flow was always more pronounced with the 2,3 isomer ($\sim$6 Å) than the 1,5 isomer (14 Å) (Table III). The extent of uncoupling by naphthalene-

Table II. Effect of Bifunctional Phenylene Malemides on the ΔpH Across the Thylakoid Membrane Under Nonphosphorylating Conditions[a]

Chloroplast treatment	Internal proton concentration (μM)	ΔpH
Control	10.0	3.00
10 μM OPBM	4.5	2.65
10 μM MPBM	5.9	2.77
10 μM PPBM	6.9	2.84
10 μM N-phenylmaleimide	10.7	3.03

[a]Thylakoids (200 μg chlorophyll/ml) were incubated for 60 s in the light with the indicated maleimides. Aliquots (0.15 ml) were mixed with an equal volume of a solution containing 20 mM Tricine/NaOH (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 2 mM $K_3Fe(CN)_6$, 0.1 mM ATP, and 0.026 mM [^{14}C]hexylamine. Hexylamine uptake was then determined and the ΔpH and internal proton concentrations calculated. ΔpH was determined to $\pm$ 0.05 pH unit.

Table III.   The Effect of Naphthalene Maleimides on Photophosphorylating Electron
Flow and the P/e_2 Ratio[a]

Thylakoid treatment	Electron flow (μeq/mg chl-hr)	Phosphorylation (μmol P$_i$/mg chl-hr)	P/e_2
Control	433	161	0.75
Naphthalene maleimide (25 μM)	357	73	0.41
1,5-Naphthalene bismaleimide (2 μM)	467	81	0.35
2,3-Naphthalene bismaleimide (2 μM)	521	73	0.28

[a]Thylakoids were treated with the naphthalene maleimides at the indicated concentrations in the
light for 1.5 min. $K_3Fe(CN)_6$ (1 mM) was the electron acceptor.

1,5-bismaleimide was similar to that observed with OPBM. These experi-
ments show that while all of these compounds are likely to cross-link, the
shorter the distance between the maleimides, the better an uncoupler the
molecule is.

To examine further the relationship between the length of the cross-
linker and the extent of uncoupling caused by the cross-link, the compound
azophenylbismaleimide (APBM) was synthesized (Fig. 3). The distance
between the maleimides in the *trans* isomer of this compound, first prepared
by Fasold *et al.* (1963), is longer (19 Å) than that of the other maleimides
used. The *trans* form of APBM predominates. This compound (5–100 μM)
caused significant inhibition of photophosphorylation only if the thylakoids
were illuminated in its presence prior to assay. Maximal inhibition (approxi-
mately 50%) was achieved at 25 μM APBM. The monofunctional analog,
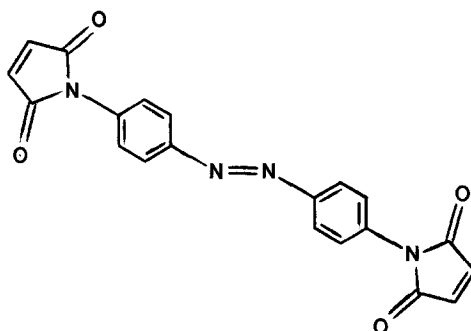*N*-(*p*-phenylazophenyl)maleimide (PAPM) inhibited at similar concentra-

Fig. 3. The structure of azophenylbismaleimide.
The molecule is shown in its *trans* form which has a
cross-linking distance of about 19 Å. By analogy to
other phenylazophenyl compounds (Erlander, 1980)
the *trans* form would be expected to be the predomi-
nant form under the conditions described here.

Table IV. The Effect of APBM and
N-(*p*-phenylazophenyl)maleimide (PAPM)
on Basal Electron Flow[a]

Thylakoid treatment	Rate of electron flow (μeq/mg chl-hr)
Control	123
1 μM APBM	145
5 μM APBM	196
10 μM APBM	181
50 μM APBM	209
5 μM PAPM	129
50 μM PAPM	141
4 μM OPBM	430

[a]Thylakoids were incubated in the presence of pyocyanine with the indicated concentration of inhibitor in the light for 90 s as described in Materials and Methods. Basal electron flow was assayed with 100 μM ATP present and 1 mM $K_3Fe(CN)_6$ as the electron acceptor.

tions. However, unlike the other bifunctional maleimides tested, thylakoids treated with APBM were only slightly, if at all, uncoupled. This can be seen in Table IV where neither APBM nor its monofunctional analog significantly stimulates nonphosphorylating electron flow. APBM forms cross-links, however, as shown in Table V. Thylakoids were treated with either *N*-ethylmaleimide, APBM, or OPBM in the dark to modify accessible sites, and the excess unreacted maleimides were removed by washing. The labeled thylakoids were then resuspended and incubated in either the light or the dark prior to assay of photophosphorylation. In the case of the bifunctional reagents, the second maleimide function can react with the group that becomes exposed in the light to form a cross-link which inhibits photophosphorylation. This inhibition was seen when either OPBM or APBM was used but

Table V. Evidence for Cross-Linking by Azophenylbismaleimide[a]

Thylakoid treatment	Preillumination	Phosphorylation (μmol/hr mg chlorophyll)	% Inhibition by light
Control	−	686	
	+	520	24%
N-ethylmaleimide	−	616	
	+	550	11%
OPBM	−	718	
	+	96	87%
APBM	−	675	
	+	336	50%

[a]Thylakoids were incubated in the dark for 5 min at room temperature with either 1 mM *N*-ethylmaleimide, 10 μM OPBM, 25 μM APBM, or in the absence of maleimides (control). The thylakoids were then washed with the buffered sucrose solution as described in Materials and Methods and then resuspended in the buffered sucrose solution. These thylakoids were then either preilluminated or kept in the dark for 2 min prior to the assay of photophosphorylation.

not in N-ethylmalemide or PAPM-treated thylakoids (not shown). In addi-
tion, the OPBM-treated thylakoids showed significant stimulation of non-
phosphorylating electron flow, while the thylakoids treated with APBM did
not (not shown). This is further evidence that although APBM can form
cross-links in the light and inhibit photophosphorylation, it is a poor uncou-
pler. This is consistent with the trend toward poorer uncoupling as the
cross-link length increases.

　　Although it is apparent that at least part of the inhibition of photophos-
phorylation by bifunctional maleimides is caused by uncoupling, it is unclear
whether the inhibition of photophosphorylation requires the modification of
the accessible thiol in addition to that exposed only in the light. MMTS reacts
rapidly with protein thiols to form a mixed disulfide consisting of the protein
cysteine group and methanethiol (Smith et al., 1975). The reagent dithiobis-
nitropyridine also reacts by a disulfide exchange mechanism. However, the
disulfide adduct formed by dithiobisnitropyridine is activated for further
exchange due to the good leaving ability of the thiopyridine group. Thus,
modification of the accessible γ subunit site with dithiobisnitropyridine
catalyzes disulfide formation within the γ subunit upon illumination of the
thylakoids (Moroney et al., 1980). A similar autocatalytic mechanism
following methanethiolation of the accessible group on the γ subunit was not
observed due to the poor leaving ability of the methanethiol moiety. However,
high concentrations of thiols added to the medium were able to cleave the
mixed protein–reagent disulfide formed by MMTS.

　　The treatment of thylakoids in the dark with MMTS, followed by
removal of the unreacted reagent by washing, prevents the inhibition of
photophosphorylation by low concentrations of OPBM. Previously (Weiss and

Table VI.　Methyl Methanethiolsulfonate Blocks the Accessible SH on the γ Subunit[a]

Thylakoid treatment	4 μM OPBM	Phosphorylation (μmol/hr mg chlorophyll)
Control	−	511
	+	168
MMTS	−	583
	+	478
MMTS + 10 mM DTT	−	629
	+	301
MMTS + 25 mM DTT	−	456
	+	196

[a]Thylakoids were incubated either with or without 20 μM MMTS in the dark for 10 min at room
temperature. The MMTS-treated thylakoids were then divided into three samples; one sample
was treated with 25 mM dithiothreitol in the dark for 5 min, and a second treated with 10 mM
dithiothreitol; no dithiothreitol was added to the third sample. All four preparations were then
washed with the buffered sucrose solution. Aliquots from each thylakoid treatment were then
illuminated in the presence or absence of 4 μM OPBM, and photophosphorylation was assayed.
DTT stands for dithiothreitol.

Table VII.   The Accessible SH of the γ Subunit Is Not Required for the Inhibition of
Photophosphorylation by N-Ethylmaleimide[a]

Thylakoid treatment	Pretreatment	Phosphorylation (μmol/hr mg chlorophyll)
Control	—	951
Control	+ NEM	245
Control	DTT	919
Control	+ NEM and DTT	236
MMTS	—	930
MMTS	+ NEM	255
MMTS	+ DTT	929
MMTS	+ NEM and DTT	305

[a]Thylakoids were incubated in the dark for 10 min at room temperature in the presence or absence of 20 μM MMTS. They were then washed with the buffered sucrose solution. Aliquots from these thylakoid preparations were then preilluminated in the presence or absence of 2 mM N-ethylmaleimide for 2 min. Where indicated, samples were then incubated for 5 min in the presence of 30 mM dithiothreitol (DTT) in the dark prior to the assay of photophosphorylation.

McCarty, 1977), it was shown that a similar treatment of thylakoids with N-ethylmaleimide blocks the light-dependent inhibition of photophosphorylation by low concentrations of OPBM. The accessible SH group on the γ subunit, necessary for cross-link formation, is blocked by N-ethylmaleimide. The incubation of MMTS-treated thylakoids, but not N-ethylmaleimide-treated thylakoids, with dithiothreitol restores the ability of low concentrations of OPBM to inhibit phosphorylation in the light (Table VI). This result indicates that the accessible SH of the γ subunit in the MMTS-treated thylakoids is regenerated by the incubation with dithiothreitol.

The incubation of thylakoids with MMTS in the dark did not inhibit photophosphorylation. This treatment also did not affect the light-dependent inhibition of phosphorylation by N-ethylmaleimide. The inhibition of photophosphorylation in MMTS-treated thylakoids incubated with N-ethylmaleimide in the light was not reversed by dithiothreitol (Table VII). This dithiothreitol treatment, which regenerates the accessible SH previously modified by the MMTS (Table VI), indicates that only the group modified by monofunctional maleimides in the light must be labeled to inhibit photophosphorylation.

Discussion

The results presented here confirm and extend our previous conclusions that bifunctional maleimides uncouple photophosphorylation. The extent of uncoupling, however, is a function of the distance between the maleimide groups. The treatment of thylakoids in the light with o-iodosobenzoate or low

concentrations of dithiobisnitropyridine may uncouple by promoting the formation of a disulfide bond in the γ subunit between the same groups cross-linked by the bifunctional maleimides (Moroney *et al.*, 1980). If so, it would be expected that this direct cross-linking of the groups would cause a more pronounced uncoupling than cross-linking by any maleimide. This was shown to be the case. The fact that the extent of uncoupling by the phenylenebismalemides is ortho > meta > para and that 1,5-naphthalenebis-maleimide is a poorer uncoupler than the 2,3-derivative indicates that the distance between the cross-linked thiol groups in the γ subunit is critical to the degree of uncoupling. The shorter the distance between the thiols, the greater the proton permeability of the membranes. Although APBM cross-links, it uncouples poorly, if at all. Thus, to cause CF_1 to assume its proton-leaky conformation, the distance between two SH groups of the γ subunit must be less than about 19 Å.

The ability of *o*-iodosobenzoate and of APBM to cross-link may indicate that the γ subunit is either very flexible or that it undergoes very large changes in its conformation when the membrane is energized. Energy-dependent changes in CF_1 have been detected by hydrogen exchange studies (Ryrie and Jagendorf, 1971, 1972) and by changes in the sensitivity of the γ subunit of CF_1 in thylakoids to proteases (Moroney and McCarty, 1982).

Cross-linking studies with the SF_1 fragment of myosin (Reisler *et al.*, 1974) have shown that two sulfhydryls in the fragment (SH-1 and SH-2) move from as far apart as 12 Å to closer than 10 Å when Mg-ADP binds to the protein (Burk and Reisler, 1977). Later, Wells and Yount (1979) showed that these groups were cross-linked both by PPBM and cobalt-phenanthroline, indicating that these sulfhydryls move at least 10 Å closer to each other when the SF_1 fragment binds ATP.

Although the experiments with MMTS show that only the group exposed to reaction with SH reagents in the light is essential for photophosphorylation, the role of this group in this process is unclear. Since the nucleotide binding sites seem to reside on the α and β subunits (Bruist and Hammes, 1981), it seems unlikely that the γ subunit is involved directly in the catalytic site. The γ subunit and its essential thiol could, however, be involved in proton translocation by the enzyme. Cross-linking within the γ subunit increases the proton permeability of thylakoids (Weiss and McCarty, 1977; Moroney and McCarty, 1979). Trypsin treatment of energized thylakoids also enhances the proton permeability of the membrane, probably by causing a clipping of the γ subunit (Moroney and McCarty, 1982).

Sulfhydryl groups within the γ subunit may also be required for the conversion of the enzyme, both free in solution and membrane bound, to its active form. A redistribution of disulfide bonds within the enzyme, similar to that which occurs when soluble CF_1 is heat treated (Ravizzini *et al.*, 1980; Andreo *et al.*, 1979), may take place when the membrane is energized.

Modification of the essential thiol in the γ subunit could prevent this disulfide–SH exchange and thereby inhibit activity.

Although the role of the γ subunit in photophosphorylation is uncertain, it is clear that modification of its structure through the cross-linking of two groups within it has profound effects on the nonproductive flow of protons through the ATPase complex of chloroplasts. Conformational changes within the γ subunit could, then, regulate the flow of protons through the membrane.

Acknowledgments

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