Cross-linking within a Subunit of Coupling Factor 1 Increases the Proton Permeability of Spinach Chloroplast Thylakoids*

MARK A. WEISS AND RICHARD E. MCCARTY

From the Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

I. The bifunctional maleimide, o-phenylenedimaleimide (OPDM), inhibits photophosphorylation in spinach chloroplast thylakoids at concentrations as low as 100 nM and is therefore, about 500-fold more effective than N-ethylmaleimide. Thylakoids must be illuminated in the presence of this reagent prior to the assay of photophosphorylation for the irreversible inhibition to be expressed. Uncouplers and adenine 5'-triphosphate plus inorganic phosphate largely prevent the development of the inhibition.

2. Phosphorylation in thylakoids treated with OPDM in the light is uncoupled from electron flow. The extent of the light-induced uptake of protons is decreased by the light and OPDM treatment, indicating that treated thylakoid membranes have enhanced permeability to protons. N,N'-Dicyclohexylcarbodiimide restores H+ uptake in OPDM-treated thylakoids.

3. N-Phenylmaleimide is only slightly more effective than N-ethylmaleimide as an inhibitor of phosphorylation. Thus, the bifunctionality of OPDM, rather than its hydrophobicity, accounts for its potency. Although incubation of thylakoids with N-ethylmaleimide in the dark does not affect the light-dependent inhibition of phosphorylation by N-ethylmaleimide, it abolishes the ability of low concentrations of OPDM to inhibit. Moreover, thylakoids incubated with OPDM in the dark followed by removal of the unreacted OPDM show inhibited rates of phosphorylation after illumination in the absence of nucleotides or sulfhydryl compounds.

4. [3H]OPDM is incorporated into the γ and ε subunits of coupling factor 1 (CF1) in thylakoids to the same extent in the light as in the dark. No other chloroplast protein was labeled to a significant extent by the OPDM. Less cysteine reacts with OPDM bound to the γ subunit of CF1, in thylakoids which had been illuminated in the presence of the OPDM than in thylakoids which had been incubated with OPDM in the dark.

5. Thus, OPDM appears to react with a readily accessible group on the γ subunit in the dark. The other maleimide function of the immobilized OPDM can then react with a group in the ε subunit of CF1, which is exposed by light-dependent conformational changes of the enzyme in thylakoids, resulting in cross-linking within the γ subunit. Since OPDM treatment in the light causes enhanced H+ permeation and uncoupling, this cross-linking may alter the conformation of CF1, to expose a proton channel.

Illumination of spinach chloroplast thylakoids in the presence of 1 mM N-ethylmaleimide promotes incorporation of this reagent (1) into the γ subunit of coupling factor 1. This incorporation, which is sensitive to nucleotides and to uncoupling agents (1), results in a partial inhibition of photosynthetic phosphorylation (2, 3). Since light induced H+ uptake (2), the magnitude of the pH differential across thylakoid membranes (4) and uncoupled electron flow (2) are unaffected by the treatment with N-ethylmaleimide, the inhibition of phosphorylation is of the energy transfer type.

Cf1, either in solution or in thylakoid membranes, contains two groups which readily react with maleimides (1, 5). These groups appear to be nonessential for photophosphorylation since their reaction with N-ethylmaleimide does not affect phosphorylation (1). One of these readily accessible groups is present in the γ subunit, whereas the other is in the ε component (1). Very little N-ethylmaleimide reacted with the α and β components and none was found in the δ subunit. Bifunctional maleimides such as the phenylenedimaleimides (6) might be expected to cross-link the γ and ε components or to cross-link the readily accessible group in the γ subunit to that exposed during illumination of thylakoids.

In this paper, we report that o-phenylenedimaleimide is a potent inhibitor of photophosphorylation. Evidence is given which suggests that OPDM forms a cross-link within the γ subunit of CF1, in thylakoids. This cross-linking alters the enzyme’s conformation in such a way that allows more rapid efflux of H+ from the thylakoids.

**MATERIALS AND METHODS**

Spinach chloroplast thylakoids were prepared from market spinach (7). Chlorophyll (8) and protein (9, 10) were estimated spectro-
photometrically. Thylakoids (0.1 mg of chlorophyll/ml) were incubated at room temperature with various concentrations of OPDM in incubation mixtures which contained: 50 mM Tricine/NaOH (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, 0.05 mM pyocyanine, and other additions as noted in figure and table legends. OPDM was dissolved in dimethylsulfoxide and aliquots added to the incubation mixtures. The final dimethylsulfoxide concentration was 0.5% or less. Dimethylsulfoxide (1%) had no effect on cyclic phosphorylation with pyocyanine. The incubation mixtures were either illuminated for the times indicated with about 2 x 10⁶ ergs/cm² of tunsten light or were kept in the dark. An amount of either diithioreitol or β-mercaptoethanol in excess of the OPDM was then added and phosphorylation and electron flow were assayed in aliquots of the incubation mixtures. The phosphorylation reaction mixtures (1.0 ml) contained 50 mM Tricine/NaOH (pH 8.0), 50 mM NaCl, 5 mM MgCl₂, 0.05 mM pyocyanine, and 0.1 mM methyl viologen and thylakoids equivalent to 20 to 50 μg of chlorophyll, and either 0.05 mM pyocyanine or 1 mM K₂Fe(CN)₆. After 1.5 to 2 min of illumination at room temperature with 2 x 10⁶ ergs/cm² of white light, trichloroacetic acid was added (10). β-Pi esterification was determined (11) in aliquots of the deproteinized mixtures. In some experiments, 3²P, was omitted and the amount of Pi, remaining was determined colorimetrically (12). Ferricyanide reduction was determined spectrophotometrically. Light-dependent H⁺ uptake (13) was estimated at pH 7.9 at 20° in incubation mixtures (2.0 ml) which contained 1 mM Tricine/NaOH, 50 mM NaCl, 0.05 mM pyocyanine, or 0.1 mM methyl viologen and thylakoids equivalent to 0.2 mg of chlorophyll. A combination pH electrode and a Heath model EU-200 electrometer were used.

To test for incorporation of radioactive OPDM into CF₁ in thylakoids, thylakoids (2 to 4 mg of chlorophyll) were incubated for 90 s at room temperature with 5 μm [³²P]OPDM (specific activity, 6 x 10⁶ cpmm/mmol) in the light (2 x 10⁶ ergs/cm²/s) or dark. The incubation mixture used was identical with that described for the development of the inhibition of phosphorylation by OPDM. The chlorophyll concentration was 0.1 mg/ml. CF₁ was isolated by extraction of the thylakoids with 0.75 mM EDTA (14) and was partially (greater than 90%) purified either by chromatography on DEAE-Sephadex A-50 (14) or by concentration followed by (NH₄)₂SO₄ precipitation (15). Prior to acrylamide gel electrophoresis in the presence of SDS, the thylakoids (15) aliquots of the CF₁ solutions were dialyzed overnight against approximately 300 volumes of a buffer which contained 20 mM Tris/HCl (pH 8.0), 2 mM ATP, and 1 mM EDTA. After fixing and staining the gels, the bands which corresponded to the CF₁ subunits were cut out and radioactivity in the bands determined (17).

o-Phenylenediaminomaleate was synthesized (18) by adding 0.12 ml of a solution which contained 28 mg of maleic anhydride (10 μmol) and about 500 μCi of (1,4-¹⁴C)maleic anhydride in warm, dry toluene to 0.2 ml of dry toluene which contained 17 mg (157 μmol) of recrystallized o-phenylenediamine. A yellow precipitate precipitated immediately. After incubation at 0–4° overnight, the product was washed several times with dry toluene. Residual toluene was removed and the product dissolved in 165 μl of N,N-dimethylformamide and 60 μl of acetic anhydride and 4 mg of sodium acetate were added. The solution was heated to 52° with stirring and the mixture was then held at 55° for 1 hr. After cooling, 36 μl of water was added slowly and a yellowish precipitate formed. The precipitate was washed several times with water to remove unreacted o-phenylenediaminomaleate and the acetate. The product, which in trial runs on this small scale was obtained on an overall yield of about 40%, co-migrated with authentic OPDM upon thin layer chromatography with Silica Gel G plates (E. Merke) developed with 1:1 (v/v) acetone/toluene. Greater than 98% of the radioactivity also co-migrated with authentic OPDM. The concentration of [¹⁴C]OPDM in solutions was determined by first titrating a constant amount of OPDM with freshly prepared cysteine, followed by estimation of the remaining cysteine with dithiobisnitrobenzoic acid (19).

OPDM and the other bifunctional maleimides used were purchased from K and K Laboratories and were recrystallized from acetone/ethanol mixtures. The recrystallized maleimides were stored at −20° over a desiccant. [1,4-¹⁴C]Maleic anhydride and [³²P]Cys were obtained from Amersham/Searle and azP₁ from ICN Life Sciences. Nucleotides were from Sigma.

RESULTS

Light-dependent Inhibition of Phosphorylation by Bifunctional Maleimides – The o-, m-, and p-phenylenediaminomaleimides were very effective inhibitors of photophosphorylation in thylakoids illuminated in the presence of these reagents prior to the assay of photophosphorylation. This is illustrated for OPDM in Fig. 1. Significant inhibition was observed with only 0.2 μM OPDM, a concentration only slightly greater than the amount of CF₁ present on the thylakoids. As is the case for the inhibition of photophosphorylation by N-ethylmaleimide (2), the inhibition by OPDM was incomplete even at very high OPDM concentrations (100 μM). Maximum inhibitions ranged from 60 to 90%. Incubation of thylakoids with OPDM in the dark had no significant effect on photophosphorylation. Washing of the thylakoids did not reverse the inhibition. Cysteine, diithioreitol, and β-mercaptoethanol prevented, but did not reverse, the inhibition by OPDM. Similar results were obtained with p-phenylenediaminomaleimide and with m-phenylenediaminomaleimide. Since OPDM seemed to be slightly more effective than the para and meta derivatives, we routinely used OPDM in our subsequent experiments. Inhibition reached its maximum extent within 60 to 90 s of illumination in the presence of 10 μM OPDM, and its half-maximal point in about 10 to 15 s.

ATP partially protects phosphorylation from inhibition by N-ethylmaleimide (2, 3) and better protection is observed when P₅ is also present (3). These reagents had quite similar effects on the development of the inhibition of phosphorylation by OPDM (Table I). Moreover, the uncoupler, NH₄Cl, partially prevented OPDM inhibition. Thus, with the exception of the fact that OPDM is approximately 500-fold more effective than N-ethylmaleimide, the properties of the development of the OPDM inhibition of phosphorylation closely resemble those of the monofunctional maleimide.

Properties of OPDM-inhibited Thylakoids – The treatment of thylakoids with N-ethylmaleimide in the light has little to no effect on the extent of proton uptake, the rate of electron flow, or the phosphorylation efficiency (2). In contrast, the extent of proton uptake supported by cyclic electron flow in thylakoids treated with OPDM in the light was inhibited (Table II). N,N'-Dicyclohexylcarbodiimide, an energy transfer inhibitor which stimulates proton uptake in CF₁-deficient
present during the illumination or not. Thus, the uncoupling OPDM reacts with a readily accessible group in CF, and a thylakoids was released regardless of whether OPDM was OPDM had been removed (Table V). Thus, the concept that dark, were illuminated despite the fact that the unreacted thylakoids, which had been previously incubated with OPDM in the light even though ATP synthesis was inhibited 74%.

Effects of OPDM treatment of thylakoids on proton uptake
Thylakoids equivalent to 2 mg of chlorophyll were incubated in the presence or absence of 5 μM OPDM in the light for 90 s. Dithiothreitol was added to a final concentration of 0.6 mM and the samples were diluted with 20 ml of a cold, buffered sucrose solution (0.4 M sucrose, 0.02 M Tricine/NaOH (pH 8.25), 0.01 M NaCl). Photophosphorylation was assayed with pyocyanine as the electron acceptor after excess OPDM was removed by addition of dithiothreitol.

| Addition          | Rate of phosphorylation | Control | μmol/mg chlorophyll |
|-------------------|-------------------------|---------|---------------------|
| Experiment I      |                         |         |                     |
| None              | 1236                    | 22.9    |                     |
| 5 μM OPDM         | 283                     | 45.7    |                     |
| OPDM + 20 μM ATP  | 155                     | 45.7    |                     |
| OPDM + 2 mM Pi    | 276                     | 22.3    |                     |
| OPDM + ATP + Pi   | 871                     | 70.5    |                     |
| Control           |                         |         |                     |

| Treatment       | Rate of phosphor- | Rate of phosphate | P/e |
|-----------------|-------------------|-------------------|------|
|                 | ylation efficiency | reduction | oxidation | ratio |
| Control         | 346               | 119               | 0.69   |
| +OPDM           | 626               | 31                | 0.16   |

Bifunctionality As Basis for Effectiveness of OPDM – The rate of reaction of maleimides with certain enzymes, including alcohol dehydrogenase (21), increases with increasing hydrophobicity of the maleimide. Since OPDM is freely soluble only in organic solvents whereas N-ethylmaleimide is quite soluble in water, OPDM is somewhat more hydrophobic than N-ethylmaleimide. However, the increased hydrophobicity of OPDM relative to N-ethylmaleimide cannot explain the extraordinary effectiveness of the bifunctional compound as an inhibitor of photophosphorylation. The monofunctional relative of OPDM, N-phenylmaleimide, which is considerably more hydrophobic than N-ethylmaleimide, was only slightly more effective than N-ethylmaleimide as a light-dependent inhibitor of photophosphorylation. Whereas half-maximal inhibition was detected with about 0.5 to 1 μM OPDM, about 500 μM N-ethylmaleimide and 200 μM N-phenylmaleimide were required to reach the same extent of inhibition. More over, the incubation of thylakoids in the dark with N-ethylmaleimide, which results in the alkylation of one group in the subunit and another in the subunit of CF1, has no effect on the light-dependent incorporation of N-ethylmaleimide into the subunit (2), abolished the ability of low concentrations of OPDM to inhibit phosphorylation in the light (Table IV).

A role for groups on Cf1 accessible to N-ethylmaleimide in the dark in the OPDM inhibition at low concentrations, suggested the possibility that the OPDM reacts with one or more of these groups in the dark or light and that the other maleimide head of the immobilized bifunctional reagent then reacts with the group(s) in CF1, exposed upon illumination of thylakoids. Since the group(s) which react with N-ethylmaleimide in the light is present on the subunit, cross-linking between the and subunits of the same CF1, molecule appeared possible. However, no evidence for cross-linking between CF1 subunits was found. Polyacrylamide gel electrophoresis patterns of CF1 from thylakoids treated with OPDM in the light were indistinguishable from those of CF1 from either untreated thylakoids or from thylakoids incubated with OPDM in the dark.

TABLE III
Uncoupling of photophosphorylation by OPDM
Thylakoids were incubated with and without OPDM as described in the legend to Table II, except that β-mercaptoethanol was used to remove the unreacted OPDM. Ferricyanide was used in the assay of photophosphorylation and was present at 1 mM.

| Thylakoid treatment | Rate of ferricyanide reduction | Rate of phosphate oxidation | P/e |
|---------------------|-------------------------------|-----------------------------|------|
| Control             | 346                           | 119                         | 0.69 |
| +OPDM               | 626                           | 31                          | 0.16 |

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thylakoids (7), restores pH uptake in OPDM-treated thylakoids to nearly the same level as control thylakoids. ATP had no effect on proton uptake in OPDM-treated thylakoids. Thus, OPDM-treated thylakoids appear to be uncoupled. This was confirmed by measurements of the phosphorylation efficiency (P/e, ratio) (Table III). The P/e, ratio in thylakoids treated with OPDM in the light was markedly reduced. Photophosphorylation linked to electron flow through each photosystem alone was also uncoupled by OPDM (not shown). Electron flow was not inhibited by OPDM. The trypsin-activated ATPase of CF1 (20) was inhibited about 20% by the incubation of thylakoids with 5 μM OPDM in the light even though ATP synthesis was inhibited 74%.

No significant release of CF1 into the medium occurred as a result of the light and OPDM treatment. Less than 2% of the trypsin- or dithiothreitol-activated ATPase activity of the thylakoids was released regardless of whether OPDM was present during the illumination or not. Thus, the uncoupling by OPDM is not a consequence of mere solubilization of CF1 which can uncouple (7). In fact, CF1 in thylakoids treated with OPDM in the light is not as readily extractable by EDTA solutions as is the enzyme in thylakoids previously incubated with OPDM in the dark.

TABLE II
Effects of OPDM treatment of thylakoids on proton uptake
Thylakoids equivalent to 2 mg of chlorophyll were incubated in the presence or absence of 5 μM OPDM in the light for 90 s. Dithiothreitol was added to a final concentration of 0.6 mM and the samples were diluted with 20 ml of a cold, buffered sucrose solution (0.4 M sucrose, 0.02 M Tricine/NaOH (pH 8.25), 0.01 M NaCl). Photophosphorylation was assayed with pyocyanine as the electron acceptor after excess OPDM was removed by addition of dithiothreitol.

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|-----------------|-------------------|-------------------|------|
|                 | ylation efficiency | reduction | oxidation | ratio |
| Control         | 346               | 119               | 0.69   |
| +OPDM           | 626               | 31                | 0.16   |

TABLE I
Effect of ATP, P, and NH4Cl on development of inhibition of photophosphorylation by OPDM
Thylakoids were incubated in the presence of 5 μM OPDM and other additions as indicated in the light as described under "Materials and Methods." Illumination of thylakoids in the presence of ATP or Pi had no effect on the rate of phosphorylation. In Experiment II, NH4Cl was removed by sedimenting the thylakoids and resuspending them in 0.4 M sucrose, 0.02 M Tricine/NaOH (pH 8.25), 0.01 M NaCl. Photophosphorylation was assayed with pyocyanine as the electron acceptor after excess OPDM was removed by addition of dithiothreitol.

| Addition          | Rate of phosphorylation | Control | μmol/mg chlorophyll |
|-------------------|-------------------------|---------|---------------------|
| Experiment I      |                         |         |                     |
| None              | 1236                    | 22.9    |                     |
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| OPDM + 2 mM Pi    | 276                     | 22.3    |                     |
| OPDM + ATP + Pi   | 871                     | 70.5    |                     |
| Control           |                         |         |                     |

| Thylakoid treatment | Control | Proton uptake | μmol/mg chlorophyll |
|---------------------|---------|---------------|---------------------|
| OPDM                | 317     | 496           | 693                 |
| +OPDM               | 199     | 199           | 579                 |
TABLE IV

Effect of treatment of thylakoids with N-ethylmaleimide in dark on inhibition of photophosphorylation by OPDM

| OPDM Photophosphorylation | Control |
|---------------------------|---------|
| µM | µmol/h/mg chlorophyll | % |
| 365 | 0.04 | 100 |
| 1 | 314 | 86 |
| 10 | 78 | 21 |

TABLE V

Light-dependent inhibition of photophosphorylation in thylakoids previously treated with OPDM in dark

Thylakoids equivalent to 2 mg of chlorophyll were incubated in the dark for 2 min at room temperature in 10 ml of a reaction mixture which contained 50 mM Tricine/NaOH (pH 8.25), 5 mM NaCl, 5 mM MgCl₂, and 5 µM OPDM. A control sample without OPDM was also run. The samples were diluted to 35 ml with the cold buffered sucrose solution described in the legend to Table II, which also contained 5 mM dithiothreitol. The suspensions were centrifuged for 15 min at 4000 x g and the pellets were resuspended in 35 ml of the buffered sucrose solution supplemented with bovine serum albumin. After centrifugation as described above, the thylakoids were resuspended in 1 ml of the buffered sucrose solution without bovine serum albumin. This procedure removes more than 99% of the unreacted OPDM. Aliquots of the suspension (50 µg of chlorophyll) were either illuminated for 90 s or kept in the dark in 0.5-ml reaction mixtures which contained no added OPDM. In some samples 0.5 mM dithiothreitol was added before the thylakoids. Photophosphorylation in control samples was unaffected by dithiothreitol or the incubations in the light or the dark. Photophosphorylation with pyocyanine as a mediator was then assayed.

Treatment of OPDM-reacted thylakoids prior to assay of phosphorylation

| Treatment of OPDM-reacted thylakoids | Photophosphorylation rate µmol/h/mg chlorophyll |
|--------------------------------------|---------------------------------------------|
| 90 s dark + dithiothreitol | 770 |
| 90 s dark no dithiothreitol | 768 |
| 90 s light + dithiothreitol | 748 |
| 90 s light no dithiothreitol | 975 |

To test for the reaction of other thylakoid components with [³⁵S]OPDM, thylakoid membranes were extracted with 80% acetone to remove pigments, were solubilized in sodium dodecyl sulfate, and subjected to electrophoresis. No polypeptides, other than the γ and ε subunits of CF₁, (as identified by their electrophoretic mobilities) contained significant amounts of radioactivity. Thus, CF₁ is by far the major thylakoid component which reacts with OPDM under these conditions.

Although indirect experiments suggested that OPDM cross-linking of the other thylakoid components with [³⁵S]OPDM, thylakoids were incubated in the light or dark in the presence of sodium dodecyl sulfate and subjected to electrophoresis. No polypeptides other than the γ and ε subunits of CF₁, (as identified by their electrophoretic mobilities) contained significant amounts of radioactivity. Thus, CF₁ is by far the major thylakoid component which reacts with OPDM under these conditions.

Incorporation of OPDM into CF₁ in Thylakoids

Thylakoids were incubated with [³⁵S]OPDM in the light or dark, and CF₁, was isolated as described under "Materials and Methods." The subunits of dissociated CF₁ were separated by electrophoresis in the presence of sodium dodecyl sulfate and the radioactivity in the subunit bands determined. The α and β components did not separate sufficiently well at the protein concentrations used in the experiment to allow the determination of the amount of radioactivity in each subunit. Very little radioactivity was found only in the regions of the gel which contained the γ and ε subunits. These findings support the concept that OPDM does not cross-link the γ and ε subunits.

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TABLE VII
Cysteine incorporation into y subunit of CF₁ in OPDM-treated thylakoids

Thylakoids (3 mg of chlorophyll) were incubated with 5 μM OPDM in the light or dark for 90 s. The unreacted OPDM was removed by washing the thylakoids. The thylakoids were suspended in 5 ml of the buffered sucrose solution described in the legend to Table II and 0.05 ml of 1 mM (1°C) cysteine (0.6 to 4.1 × 10⁻⁶ cmol/nmol) added. After 10 min in the dark at 0°C, 30 ml of the buffered sucrose solution was added and the thylakoids sedimented at 7000 × g for 10 min. CF₁ was then isolated and its subunits separated on sodium dodecyl sulfate-polyacrylamide gels run in triplicate in the presence of β-mercaptoethanol. The incorporation of cysteine into a plus β subunits was 0.02 mol/mol of CF₁ and that into the δ subunit was < 0.01 mol/mol. Cysteine was incorporated into the ε subunit, to about 0.1 to 0.2 mol/mol of CF₁. No cysteine was incorporated into CF₁ from thylakoids which had not been incubated with OPDM.

| Experiment | Cysteine incorporation into y subunit of CF₁ in thylakoids treated with OPDM |
|------------|--------------------------------------------------------------------------------|
| Light OPDM-treated | Dark OPDM-treated |
| 1 | 0.58 | 0.69 |
| 2 | 0.59 | 0.69 |
| 3 | 0.57 | 0.73 |

**Fig. 2.** Photophosphorylation as a function of CF₁ lost from the thylakoids. Aliquots (0.5 ml) of a thylakoid suspension (2 mg of chlorophyll/ml) were diluted into 10 ml of cold 1 mM EDTA as well as into separate 10 ml aliquots of 1 mM EDTA which contained 2, 3, 4, and 5 mM NaCl. After 10 min at 0°C, the suspensions were centrifuged at 26,000 × g for 30 min. The pellets were resuspended in a minimal volume of the buffered sucrose solution described in the legend to Table II. Photophosphorylation with pyocyanine as the mediator of electron flow was assayed. The Ca²⁺-ATPase of the thylakoids (50 μg of chlorophyll) was activated with trypsin (250 μg/50 μg of chlorophyll) and assayed as described (14). The maximum rate of phosphorylation (1 mM EDTA plus 5 mM NaCl sample) was 915 μmol/h/mg of chlorophyll. The ATPase activity of this sample was 251 μmol/h/mg of chlorophyll.

**DISCUSSION**

Bifunctionality appears to be the key to the extraordinary effectiveness of OPDM as an inhibitor and uncoupler of photophosphorylation. Like its monofunctional relative, N-ethylmaleimide, OPDM can react with a group in the γ subunit of CF₁ in thylakoids which is accessible in the dark and another group which becomes accessible in the light. During the period (about 10 to 30 s) between the addition of thylakoids to incubation mixtures containing OPDM and illumination, OPDM reacts with the accessible group on the γ subunit of CF₁. The second maleimide functional group is thus immobilized in a position where it can rapidly react with the group in CF₁, which becomes accessible upon illumination. This mechanism can account for the observation that bifunctional maleimides are much more potent inhibitors than monofunctional ones since the effective concentration of maleimide would be increased dramatically by its immobilization on the γ subunit.

Another reason for the effectiveness of OPDM as an inhibitor of phosphorylation is that, unlike N-ethylmaleimide, OPDM uncouples photophosphorylation and increases the proton permeability of the membrane. The development of the inhibition of phosphorylation by N-ethylmaleimide is very dependent on the magnitude of the pH differential (ΔpH) across the membrane (4). In all likelihood, this result means that the conformational change in CF₁, which exposes a critical residue to reaction with maleimides, occurs only at high ΔpH values. The cross-linking by OPDM is thus a self-limiting reaction, since as the cross-links are made ΔpH is decreased and the conformation of CF₁ relaxes.

The groups cross-linked in CF₁, in illuminated thylakoids are likely to be —SH groups and must be close to each other (< 10 Å). Andre0 and Vallejos (22) reported that the sulfhydryl reagent, 2,2'-dithiobis(5-nitropyridine) inhibits photophosphorylation in a manner similar to N-ethylmaleimide (22). Oxidation of neighboring sulfhydryl groups to a disulfide is a plausible explanation for the inhibition by the dithiobisnitropyridine reagent. OPDM could cross-link these same groups.

The fact that cross-linking modifies the proton permeability of thylakoid membranes is most interesting, because it suggests a role for the γ subunit in transmitting the energy of the pH differential to the active sites of CF₁, which are present on the larger α or β subunits (23). Significantly, only the ε, δ, and γ subunits of the coupling factor-ATPase of a thermophilic bacterium are required to restore proton impermeability to phospholipid vesicles which contain the more hydrophobic components of the ATPase complex of this organism (24). The cross-linking may modify the structure of the γ component of CF₁ in such a way as to allow protons to escape from the interior of the thylakoids through the entire ATPase complex even though ATP synthesis is not taking place. Cross-linking could also modify the way in which CF₁ interacts with the thylakoid membrane and result in the exposure of a proton channel through the membrane. Wagner and Junge (25) have shown that a fast phase of internal proton release following a single turnover flash is virtually eliminated by the OPDM and light treatment, but that the slow phase is unaffected. They proposed that the OPDM modified CF₁, is triggered into a conformation which allows rapid proton conduction across the thylakoid membrane. This conformation relaxes very quickly after passage of protons.

Finally, the role for the group in the γ subunit of CF₁, in illuminated thylakoids which is alkylated by maleimides in the mechanism of photophosphorylation remains an intriguing question. Protonation and deprotonation of the group could be involved in either essential conformational changes in CF₁ or in the transport of protons to the active site of ATP synthesis.

**Acknowledgment—**We are grateful for the efforts of Mr. Gary Wilton who participated in the early stages of this investigation.
Cross-linking of CF

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M A Weiss and R E McCarty

J. Biol. Chem. 1977, 252:8007-8012.

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