Uncoupling and Energy Transfer Inhibition of Photophosphorylation by Sulfhydryl Reagents*

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The action of the sulfhydryl reagents, o-iodosobenzoate and 2,2'-dithiobis(5-nitropyridine), on photophosphorylation by spinach chloroplast thylakoids has been reevaluated. Both of these compounds were previously reported to be energy transfer inhibitors of photophosphorylation, provided the thylakoids were illuminated in their presence prior to assay. We show here that the treatment of thylakoids in the light with iodosobenzoate uncouples phosphorylation from electron flow. This treatment enhances nonphosphorylating electron transport and markedly decreases the efficiency of photophosphorylation. The light-induced transmembrane pH gradient is also diminished by exposure of thylakoids to iodosobenzoate in the light. Dithiobisnitropyridine has been found to act either as a light-dependent uncoupler or energy transfer inhibitor. At low concentrations of this reagent, illumination elicits uncoupling, whereas at higher concentrations, energy transfer inhibition is induced. The uncoupling by iodosobenzoate and by low concentrations of dithiobisnitropyridine is largely prevented by the prior incubation of thylakoids with N-ethylmaleimide in the dark. Under these conditions, N-ethylmaleimide was previously shown to react with a group on the γ subunit and with a group on the ε subunit of coupling factor 1.

The effects of these sulfhydryl reagents on photophosphorylation are compared to those of maleimides, and a model for the inhibition of phosphorylation by these reagents is proposed. Cross-linking two sulfhydryl groups within the γ subunit of coupling factor 1, either directly by disulfide bond formation or by bifunctional maleimides, causes thylakoids to become proton-leaky and phosphorylation is uncoupled from electron flow. In contrast, modification of a sulfhydryl, which becomes exposed only in the light, by monofunctional reagents elicits energy transfer inhibition.

Photophosphorylation, the light-dependent synthesis of ATP by illuminated chloroplasts, may be inhibited in a number of ways. Reagents that block electron transport also inhibit ATP synthesis since the generation of the transmembrane electrochemical gradient, the driving force for ATP synthesis,

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is dependent upon electron flow. Chemicals or treatments that increase the proton permeability of chloroplast thylakoid membranes uncouple phosphorylation from electron flow. By decreasing the proton gradient, uncoupling agents inhibit ATP synthesis but allow electron transport to occur at high rates. In contrast, direct inhibitors of phosphorylation, often called "energy transfer inhibitors," block both phosphorylation and that portion of the electron flow that is a consequence of proton efflux linked to phosphorylation (see Ref. 1 for a discussion of inhibition of photophosphorylation).

Reagents that attack −SH groups, including N-substituted maleimides (2-4), iodosobenzoate (5), and dithiobisnitropyridine (6), partially inhibit photophosphorylation when chloroplast thylakoids are illuminated in their presence prior to the assay of photophosphorylation. No inhibition is observed when the incubation is carried out in the dark. Adenine nucleotides and uncoupling agents protect phosphorylation from inhibition by these reagents.

Bifunctional maleimides are more effective inhibitors than monofunctional ones. o-Phenylenedibismaleimide (3) and dithiobisethylmaleimide (7) cross-link two groups within the γ subunit of coupling factor 1 (CF1) in illuminated thylakoids. Two groups in the γ subunit also react with N-ethylmaleimide in the light (8). Yet, the manner in which phosphorylation is inhibited by monofunctional maleimides differs from that of bifunctional maleimides. N-Ethylmaleimide acts as a phosphorylation or energy transfer type inhibitor (2). In contrast, o-phenylenedibismaleimide (3) and dithiobisethylmaleimide (7) act at least in part as uncouplers. The uncoupling effect of the latter bifunctional compound is reversed by thiols since they reduce its disulfide bond, breaking the cross-link.

In view of the fact that bifunctional maleimides produce a light-dependent uncoupling, the observation that iodosobenzoate (5) and dithiobisnitropyridine (6) act as light-dependent energy transfer inhibitors was surprising. These reagents can oxidize vicinal thiols to disulfides (9). The same thiols in the γ subunit of CF1,1 cross-linked by the bifunctional maleimides could be oxidized to disulfides by iodosobenzoate or dithiobisnitropyridine. In this paper, we report that iodosobenzoate acts as an uncoupler of photophosphorylation and that dithiobisnitrobenzoate can act either as an uncoupler or as an energy transfer inhibitor, depending on the conditions of illumination.

MATERIALS AND METHODS

Spinach chloroplast thylakoids were prepared from market spinach as described previously (10) except that in some experiments the homogenate was filtered through nylon mesh and the thylakoids were collected by centrifugation at 6000 × g for 1 min. Iodosobenzoate,

1 The abbreviations used are: CF1, coupling factor 1; Tricine, N-Tris(hydroxymethyl)methylglycine.

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2,2′-dithiobis(5-nitropyridine), N-ethylmaleimide, and o-phenylene-
bismaleimide were from Sigma. Chlorophyll (11) and photophos-
phorylation (12) were determined by published methods. Ferricyanide
reduction was determined spectrophotometrically (13) and oxygen
reduction was determined in the presence of methyl viologen with a
Clark type oxygen electrode. Transmembrane pH gradients (ΔpH)
were estimated from hexylamine distributions. [14C]Hexylamine up-
take was estimated by silicone fluid centrifugal filtration as described
previously (14) except that [14C]hexylamine was omitted and the entire
glycerol-trichloroacetic acid layer was taken for determination of
radioactivity. Thylakoids (20 to 100 µg of chlorophyll/ml) were
treated in white light (usually about 0.25 watts/cm²) or in the dark
for 90 s in a mixture containing 50 mM Tricine/NaOH (pH 8.0),
50 mM NaCl, 5 mM MgCl₂, either
mM methyl viologen, and sulfhydryl reagent at the concentrations
indicated in figure and table legends. In some experiments, the treated
thylakoids were collected by centrifugation at 5000 × g for 5 min and
and were washed in a buffered sucrose solution containing 0.4 M sucrose,
0.02 M Tricine/NaOH (pH 8.0), and 0.01 M NaCl. Bovine serum
alubum (0.1%) was added to the buffer solutions in experiments in
which thylakoids were treated with dithiobisnitropyridine to facilitate
the removal of the noncovalently bound reagent. All reaction mix-
tures contained 4 mM ADP and 8 mM potassium phosphate buffer (pH 8.0),
containing about 10⁸ cpm of [3H]pyocyanine or 0.025 mM pyocyanine) and thylakoids equivalent
to 20 to 50 µg of chlorophyll/ml. NaCN (1 mM) was present if methyl
viologen was the electron acceptor. In photophosphorylation experi-
ments, 3 mM ADP and 2 mM potassium phosphate buffer (pH 8.0),
containing about 10⁶ cpm of [3H]pyocyanine was added. Nonphosphory-
lating (basal) electron flow was assayed in the presence of 0.1 mM ATP.

**RESULTS**

**Uncoupling of Photophosphorylation by Iodosobenzoate—**

The conclusion (5) that iodosobenzoate treatment of thyla-
koids in the light causes energy transfer inhibition of photo-
phosphorylation was based primarily on the observation that
this treatment inhibited phosphorylating electron flow. In
more than 10 experiments, however, we were unable to obtain
strong inhibition of phosphorylating electron flow from water
to methyl viologen. The inhibition by 1 mM iodosobenzoate
ranged from 0 to 20%, even though phosphorylation enhanced
electron flow by 2-fold or more. This slight inhibition is likely
due to a direct effect of iodosobenzonzoate on the assay of electron
flow. Iodosobenzoate is reduced by illuminated thylakoids at
a rate of 22 µmol h⁻¹ mg of chlorophyll⁻¹. Moreover, removal of the
untreated iodosobenzoate by washing reverses the inhibition of electron flow but not that of photophosphory-
lization (Table I). Uncoupled, phosphorylating, and nonphos-

**TABLE I**

| Iodosobenzoate during illumination | Further treatment | Rate of electron transport | Rate of phosphorylation | P/e |
|----------------------------------|-------------------|---------------------------|-------------------------|-----|
|                                  | +                 |                           |                         |     |
|                                  | Wash              |                           |                         |     |
|                                  | Wash              |                           |                         |     |

**TABLE II**

| Thylakoid treatment | Dithiobenzoate | Ferricyanide reduction rate | Photophosphorylation rate | P/e |
|---------------------|----------------|----------------------------|--------------------------|-----|
| No iodosobenzoate   | 230            | 68                         | 0.59                     |     |
| No iodosobenzoate   | +              | 263                        | 88                       | 0.67|
| Plus iodosobenzoate | 255            | 12                         | 0.09                     |     |
| Plus iodosobenzoate | +              | 252                        | 73                       | 0.58|

**TABLE III**

| Thylakoid treatment | ΔPH |
|---------------------|-----|
| No iodosobenzoate, dark | 3.16 | 2.76 |
| No iodosobenzoate, light | 3.15 | 2.81 |
| Plus iodosobenzoate, dark | 3.21 | 2.80 |
| Plus iodosobenzoate, light | 7.78 | 2.69 |

Fig. 1. Effects of iodosobenzoate on electron transport. Thyla-
koids (100 µg of chlorophyll) were illuminated for 60 s in the
presence of iodosobenzoate at the indicated concentrations in 1 ml of
preillumination mixture that contained 15 µM pyocyanine and 1 mM
NaCN. Methyl viologen was then added to 300 µM and electron
transport was determined by oxygen uptake in the presence of either
0.1 mM ATP (basal), 2 mM ADP and 2 mM P₃ (coupled), or 0.1 mM
ATP + 10 mM methylamine (uncoupled). Rates are expressed as amol
of O₂ consumed h⁻¹ mg of chlorophyll⁻¹.
phorylating electron transports are inhibited by iodosobenzoate, especially at high concentrations (Fig. 1). Thus, it appears that iodosobenzoate and light treatment does not specifically inhibit phosphorylating electron flow.

Whether or not iodosobenzoate was present during the assay, the illumination of thylakoids in its presence prior to assay enhanced the rate of basal (nonphosphorylating) electron flow (Fig. 1). This stimulation occurs at concentrations of the reagent that also cause inhibition of photophosphorylation (5). Thus, the iodosobenzoate and light treatment of thylakoids appears to uncouple. This conclusion is confirmed by the observation that this treatment causes a marked decrease in the phosphorylation efficiency (P/e ratio), as shown in Table II. As expected, dithiothreitol largely reversed the inhibition of phosphorylation.

Uncoupling by the incubation of thylakoids with iodosobenzoate

Table IV

TREATMENT OF THYLAKOIDS IN THE DARK WITH N-ETHYLMALEIMIDE PROTECTS PHOSPHORYLATION FROM INHIBITION BY O-IODOSOBNZOATE

Thylakoids (0.1 mg of chlorophyll/ml) were incubated for 5 min at room temperature in the dark in the presence of 50 mM Tricine/NaOH (pH 8.0), 5 mM MgCl₂, 50 mM NaCl, and with or without 2 mM N-ethylmaleimide. The thylakoids were collected by centrifugation and washed in the buffer sucrose solution. The control and NaOH (pH 8.0), 5 mM MgCl₂, 50 mM NaCl, and with or without 1 mM iodosobenzoate for 30 s were illuminated in the presence of the indicated concentrations of the mediator of electron flow. Phosphorylation was assayed for 90 s in the presence of methyl viologen (0.5 mM).

| Thylakoid treatment     | Iodosobenzoate in preillumination (μmol/h/mg chlorophyll) | Photophosphorylation (μmol ADP/mg chlorophyll) |
|-------------------------|----------------------------------------------------------|-----------------------------------------------|
| Incubated in the dark minus N-ethylmaleimide | - | 192 |
| Incubated in the dark minus N-ethylmaleimide   | + | 33 |
| Incubated in the dark plus N-ethylmaleimide    | - | 185 |
| Incubated in the dark plus N-ethylmaleimide    | + | 145 |

**Fig. 2. Uncoupling and energy transfer inhibition by dithiobisnitropyridine.** Thylakoids (20 μg of chlorophyll, 0.5 ml) were illuminated in the presence of the indicated concentrations of dithiobisnitropyridine for 60 s. Pyocyanine (10 μm) was the mediator. A reaction mixture (0.5 ml) which contained 50 mM Tricine/NaOH, 50 mM NaCl, 5 mM MgCl₂, 4 mM ADP, 4 mM potassium phosphate buffer containing about 1 μCi of ³²P, and 2 mM K₃Fe(CN)₆ was then added and the mixtures were illuminated for 3 min. Electron transport was determined by assay of the disappearance of ferricyanide. In the experiment shown in A, control thylakoids were used. In that shown in B, thylakoids (2 mg of chlorophyll/ml) were incubated for 10 to 15 min in the dark at 0°C in the presence of 2 mM N-ethylmaleimide. The photophosphorylation electron flow; A = A, basal electron flow. Rates are expressed as μmol of ferricyanide reduced h⁻¹ mg of chlorophyll⁻¹.
Inhibition of Photophosphorylation

iodosobenzoate in the light largely prevents iodosobenzoate uncoupling. This treatment also prevents uncoupling by low concentrations of bifunctional maleimides (3, 7) which cross-link a maleimide-reactive accessible group on the \( \gamma \) subunit to one that becomes reactive in the light. In view of the fact that \( N \)-ethylmaleimide treatment blocks the inhibition by iodosobenzoate and by bifunctional maleimides, it seems likely that disulfide bond formation in the \( \gamma \) subunit is the cause of the light-dependent inhibition of ATP synthesis by iodosobenzoate.

Uncoupling and Energy Transfer Inhibition by Dithiobisnitropyridine—Dithio compounds can form mixed disulfides between the compound and a protein thiol or oxidize vicinal thiols to disulfides (17). By analogy to the action of iodosobenzoate and bifunctional maleimides on one hand, and monofunctional maleimides on the other, the formation of a disulfide in \( \gamma \) should uncouple, whereas the formation of a mixed disulfide should give energy transfer inhibition. Depending on the concentration of dithiobisnitropyridine present, either uncoupling or energy transfer inhibition is elicited by illumination (Fig. 2A). Low concentrations strongly uncouple, giving rise to high rates of electron flow and severely inhibited ATP synthesis. At higher concentrations, phosphorylation was actually less inhibited and electron transport was slowed to near the basal level. The pretreatment of thylakoids with \( N \)-ethylmaleimide prevented the uncoupling but not the energy transfer inhibition by dithiobisnitropyridine (Fig. 2B). Both uncoupling and energy transfer inhibition by this reagent require illumination for their onset and are reversed by illumination of thylakoids in the presence of 10 mM dithiothreitol (not shown). Basal electron flow was strongly stimulated by low concentrations of dithiobisnitropyridine, but as previously reported (6), higher concentrations have little effect (Fig. 2A).

These results suggest that dithiobisnitropyridine may form a disulfide bond in the \( \gamma \) subunit at low concentration and mixed disulfides at higher concentration. Phosphorylation by thylakoids that had been incubated with dithiobisnitropyridine in the dark, followed by removal of the excess reagent by washing, becomes inhibited when the thylakoids are illuminated (not shown). The reagent probably reacts in the dark with the accessible thiol on the \( \gamma \) component. In the light, another thiol on the \( \gamma \) subunit may approach this mixed disulfide and displace the reagent, resulting in the formation

| Thylakoid treatment | Rate of phosphorylation | Rate of electron flow | P/\( \varepsilon \) |
|---------------------|-------------------------|----------------------|----------------|
| +DTNP | Dark | 139 | 336 | 0.82 |
| +DTNP | Light | 13 | 553 | 0.05 |
| +DTNP | 2 mM MalNEt, light | 56 | 371 | 0.30 |
| -DTNP | 2 mM MalNEt, light | 95 | 347 | 0.55 |
| +DTNP | 0.1 mM DTNP, light | 28 | 187 | 0.30 |
| -DTNP | 0.1 mM DTNP, light | 31 | 187 | 0.32 |

**Table V**

**Partial prevention of light dependent uncoupling by dithiobisnitropyridine**

Thylakoids (20 \( \mu \)g of chlorophyll) were first incubated for 60 s in the dark in 0.5 ml of an incubation medium containing 10 \( \mu \)M pyocyanine and in the presence or in the absence of 2 \( \mu \)M dithiobisnitropyridine. Where indicated, either \( N \)-ethylmaleimide or dithiobisnitropyridine was rapidly added to give final concentrations of 2 and 0.1 mM, respectively, and the samples were immediately given a second 60-s incubation in the light or in the dark. Phosphorylation and electron flow were assayed for 3 min in the presence of K\( _3 \)Fe(CN)\( \text{6} \). Phosphorylation and electron flow rates are given as pmol of P\( \text{1 esterified or of Fe(CN)} \text{6: reduced, respectively, h}^{-1} \text{ mg of chlorophyll}^{-1} \). DTNP, dithiobisnitropyridine; MalNEt, \( N \)-ethylmaleimide.

Fig. 3. A model for the inhibition of photophosphorylation by iodosobenzoate, dithiobisnitropyridine, and maleimides. IBZ, iodosobenzoate; NPSNP, dithiobisnitropyridine; NEM, \( N \)-ethylmaleimide; MAL, \( \alpha \)-MAL, o-phenyleneisobenzamide. Cross-linking within \( \gamma \), either by formation of a disulfide (iodosobenzoate or low concentrations of dithiobisnitropyridine or by bifunctional maleimides) causes uncoupling. Modification of a sulfhydryl in \( \gamma \) by \( N \)-ethylmaleimide or dithiobisnitropyridine present in the medium at high \( \gamma \) concentrations generates energy transfer inhibition.
of an intrapeptidic disulfide. When high concentrations of dithiobisnitropyridine are present in the medium, the reagent may react with the thiol that is exposed in the light to form a mixed disulfide more rapidly than the reaction of this thiol with the mixed disulfide between the reagent and the accessible thiol. The observation (Table V) that uncoupling is partially prevented when thylakoids, previously treated with 2 μM dithiobisnitropyridine in the dark, were incubated in the light with either 100 μM dithiobisnitropyridine or 2 mM N-ethylmaleimide is in accord with this proposal.

**DISCUSSION**

We can now arrive at a general model for the action of sulphydryl reagents on photophosphorylation. Our interpretation of how iodosobenzoate and dithiobisnitropyridine inhibit is shown in Fig. 3. Two —SH groups on the γ subunit of CF, are shown. One is accessible to attack by —SH reagents in the dark, whereas the other is exposed to reaction only in the light. Modification of the accessible group does not inhibit ATP synthesis. In contrast, the reaction of the group that became accessible in the light causes an energy transfer type of phosphorylation inhibition. However, when these —SH groups are cross-linked, either by disulfide bond formation or by bifunctional maleimides, uncoupling occurs. In this discussion, we will assume that iodosobenzoate and dithiobisnitropyridine exert their effects through their interaction with —SH groups on the γ subunit of CF. This assumption is reasonable in view of the close similarity between these reagents and maleimides in the manner in which they inhibit photophosphorylation. The development of the inhibition of phosphorylation by these reagents requires light and is prevented by nucleotides and uncouplers. Moreover, the reaction of a group in the γ subunit with N-ethylmaleimide in the dark prevents light-dependent uncoupling by bifunctional maleimides (3, 7) and by iodosobenzoate and dithiobisnitropyridine. Mono- (8) and bifunctional (3, 7) maleimides react with groups in γ. Finally, iodosobenzoate and light treatment of thylakoids causes the formation of a new disulfide in the γ as well as one in the β subunit (16).

Bifunctional maleimides, iodosobenzoate, and low concentrations of dithiobisnitropyridine are light-dependent uncouplers of photophosphorylation. Bifunctional maleimides partially cross-link two groups in the γ subunit and this cross-linking causes increased proton permeability and, therefore, uncoupling. Recently, α-, m-, and p-phenylenebismaleimides were found to act as light-dependent energy transfer inhibitors at high concentrations. This inhibition is not alleviated by the prior treatment of thylakoids with N-ethylmaleimide in the dark. When these bifunctional maleimides are present in high concentration (>20 μM), they probably react with the group in the γ subunit which is exposed in the light more rapidly than the maleimide group bound to the accessible group in the γ component. Therefore, cross-linking would not take place under these conditions. Iodosobenzoate or low concentrations of dithiobisnitropyridine probably cause the formation of a disulfide bond within the γ subunit. N-Ethylmaleimide blocks the uncoupling by these reagents and by bifunctional maleimides, suggesting that the accessible —SH group on the γ subunit is likely to be part of this disulfide. Since this subunit contains three (18) or four —SH groups (19) and since the specificity of maleimides for —SH groups is not absolute, we cannot conclude that bifunctional maleimides cross-link the same —SH groups that form the inhibitory disulfide bond. Further experiments, including peptide mapping of the γ subunit, which is in progress in the Ithaca laboratory, will be required to establish which groups react. Nonetheless, the possibility that the same groups are involved in cross-linking and disulfide bond formation is intriguing. p-Phenylenbismaleimide (3) is an effective inhibitor of photophosphorylation and provides a cross-link span of 12 to 14 Å. It would be remarkable if the same —SH groups were directly cross-linked by disulfide bond formation since the consequences of these two kinds of cross-linking appear to be so similar.

Although cross-linking within the γ subunit clearly uncouples, reaction of presumably the same —SH groups in the γ subunit with monofunctional reagents causes predominately an energy transfer inhibition. Monofunctional maleimides, including N-ethylmaleimide (2, 4) and even the bulky N-naphthylmaleimide are largely energy transfer inhibitors. Dithiobisnitropyridine, at high concentrations, may form mixed disulfides with the accessible —SH and with that which is exposed by illumination. The possibility that this reagent causes the formation of disulfide bonds elsewhere in CF, cannot be ruled out at present. How modification of an —SH group(s) in the γ component by monofunctional reagents inhibits phosphorylation is not understood. In view of the observation that cross-linking groups in the γ subunit affects proton permeability of thylakoid membranes (3, 7), this group(s) may play a role in proton translocation by the ATPase complex. Moreover, disulfide interchange may be required to convert CF in thylakoids to a form that is active in photophosphorylation. Heat treatment of soluble CF2 activates Ca2+-ATPase (20) and causes the formation of a disulfide bond in the α subunit with monofunctional reagents, indicating that the accessible "SH group(s) is part of this disulfide. It would be remarkable if the same "SH groups were directly involved in cross-linking and disulfide bond formation. 

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