

## SULPHYDRYL GROUPS IN PHOTOSYNTHETIC ENERGY CONSERVATION: FURTHER EVIDENCE OF VICINAL DITHIOLS INVOLVEMENT SHOWN BY LIGHT-DEPENDENT EFFECTS OF *o*-IODOSOBENZOATE

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### 1. Introduction

The role of sulphhydryl groups in photosynthetic phosphorylation has been an open question ever since the early observations [1] that this process was sensitive to reagents such as *p*-chloromercuribenzoate. The spinach chloroplasts coupling factor CF<sub>1</sub> which presumably participates in the catalysis of photophosphorylation is made up of five different subunits [2] and contains a total of 12 half-cystine residues [3]: there are eight free sulphhydryl groups and two disulfide bounds.

Incubation of chloroplasts in the light with *N*-ethylmaleimide resulted in inhibition of photophosphorylation [4] and in the incorporation of tritium-labeled reagent into the  $\gamma$  subunit of CF<sub>1</sub> [5].

We have recently found that 2, 2'-dithio bis-(5-nitropyridine) (DTNP) inhibited ATP synthesis and hydrolysis in chloroplasts preilluminated in the presence of this sulphhydryl reagent [6]. Reversal of the inhibition was obtained by a second preillumination in the presence of thiol groups. From quantitative determinations of the DTNP disappeared and of the thione formed it was deduced that the reagent had oxidized vicinal dithiols exposed by a light-induced conformational change of membrane-bound CF<sub>1</sub>.

In order to confirm this suggestion we decided to study the effect of *o*-iodosobenzoate in chloroplasts in experimental conditions similar to those described for DTNP [6]. *o*-Iodosobenzoate is one of the most selective oxidant reagent for sulphhydryl groups [7]. We report here that *o*-iodosobenzoate inhibits ATP synthesis and hydrolysis and coupled electron transport

in spinach chloroplasts in a light-dependent fashion and with kinetics very similar to those DTNP. These results are related to the oxidation by the reagent of vicinal dithiols exposed by light.

### 2. Experimental

Chloroplasts were isolated from spinach leaves (*Spinacea oleracea* L) as described [8] and suspended in 250 mM sucrose, 20 mM Tris-HCl (pH 7.8) and 5 mM MgCl<sub>2</sub>. Preincubations of chloroplasts (50  $\mu$ g of chlorophyll) with *o*-iodosobenzoate in dark or light for 1 min were carried out in 0.5 ml of the same medium plus 50  $\mu$ M pyocyanine. Total chlorophyll [9], cyclic photophosphorylation, electron transport and the trypsin-activated Ca-ATPase [6] were determined as described. Dio-9, FCCP, discarine B and X-537 A were generous gifts from Dr P. L. Hoogland, Gis-Brocades N.V. (Delft, The Netherlands), Dr P. G. Heytler, E. I. Du Pont de Nemours & Co (Wilmington, USA), Dr O. A. Mascaretti (Departamento de Química Orgánica, Universidad de Buenos Aires) and Dr J. Berger, Department of Chemical Research, Hoffman-La Roche Inc., New Jersey.

### 3. Results

Preincubation of spinach chloroplasts in the light for 1 min with *o*-iodosobenzoate in the presence of pyocyanine and MgCl<sub>2</sub> resulted in the inhibition of photophosphorylation and of trypsin-activated Ca-

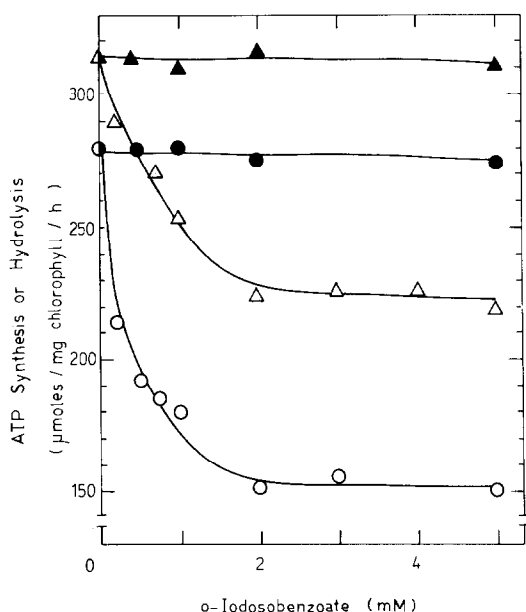


Fig.1. Effect of preincubation of chloroplasts with *o*-iodosobenzoate on cyclic photophosphorylation and on the trypsin activated, Ca-ATPase. Preincubation of chloroplasts with the stated concentrations of *o*-iodosobenzoate in the dark (closed symbols) or in the light (open symbols) and photophosphorylation (●,○), and ATPase (▲,△) activities determinations were carried out as described in the text.

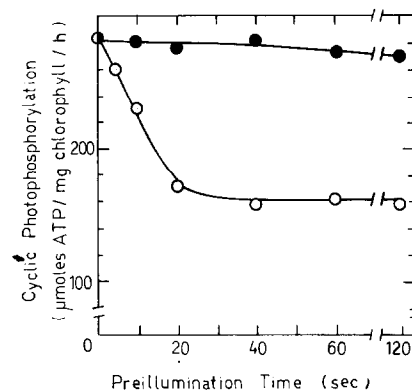


Fig.2. Kinetics of *o*-iodosobenzoate inhibition of photophosphorylation. Chloroplasts were preincubated in the light without (●—●) or with 2 mM *o*-iodosobenzoate (○—○) for the time stated.

ATPase activity (fig.1). None of these activities was affected by preincubation of chloroplasts with 5 mM (or even higher concentrations) of *o*-iodosobenzoate in the dark. The titration curves of the light-dependent inhibition were similar in both cases. About 2 mM *o*-iodosobenzoate gave the maximum effect which was near fifty and thirty per cent inhibition in photophosphorylation and ATPase activity, respectively (fig.1).

Fig.2 shows that, with 2 mM *o*-iodosobenzoate the

Table 1  
Prevention of *o*-iodosobenzoate inhibition by adenine nucleotides and uncouplers

Expt.	Additions to the pre-illumination stage	Cyclic photophosphorylation (μmoles ATP/mg chlorophyll/h)	
		Controls	<i>o</i> -Iodosobenzoate-treated chloroplasts
1	None	395	188 (52)
	20 μM ADP	390	275 (29)
	20 μM ADP + 2 mM P <sub>i</sub>	393	373 (5)
	20 μM ATP	394	299 (24)
	20 μM ATP + 2 mM P <sub>i</sub>	387	390 (0)
	2 mM P <sub>i</sub>	393	196 (50)
2	None	420	250 (40)
	5 mM NH <sub>4</sub> Cl	417	420 (0)
	2 μM FCCP	372	355 (5)
	200 μM Discaraine B	358	201 (44)
	Dio-9 (5 μg/ml)	346	213 (38)

When added, in the preillumination stage, *o*-iodosobenzoate was 2 mM. Experimental conditions were as described in the text. Numerals in parentheses are per cent of inhibition.

Table 2  
Effect of preincubation of chloroplasts with *o*-iodosobenzoate in the light  
on water to methylviologen electron transport

Additions	Electron transport ( $\mu\text{moles O}_2/\text{mg chlorophyll/h}$ )	
	Controls	<i>o</i> -Iodosobenzoate-treated chloroplasts
None	135	138
2 mM ADP + 2 mM $\text{P}_i$	254	142
2 mM ADP + 2 mM $\text{P}_i$ + 5 $\mu\text{M}$ X-537 A	288	287

Experimental conditions were as described in the text. *o*-Iodosobenzoate was 2 mM when added in the preillumination of chloroplasts. It has no effect in dark preincubations. The ionophore X-537 A was used as an uncoupler [21].

plateau of photophosphorylation inhibition was reached in 40 s. The halftime was about 8 sec.

The inhibition by *o*-iodosobenzoate was partially prevented by low concentrations of ADP or ATP present during the preincubation (table 1, expt. 1). Higher concentrations of the nucleotides (2 mM) gave the same result. However, complete prevention was achieved by low (or high) concentrations of ADP or

ATP plus 2 mM  $\text{P}_i$ . The latter alone was ineffective. Uncouplers like  $\text{NH}_4\text{Cl}$  or FCCP also totally prevented the inhibition (table 1, expt. 2), while energy transfer inhibitors like discarine B [10] or Dio-9 [11] were without effect.

Preincubation of chloroplasts in the light with 2 mM *o*-iodosobenzoate affected neither the basal electron transport from water to methylviologen nor the uncoupled activity but diminished the rate of the coupled electron transport to the basal level (table 2). Thus, *o*-iodosobenzoate acts as an inhibitor of energy transfer.

Reversal of *o*-iodosobenzoate effect can be obtained with a second illumination of chloroplasts in the presence of dithioerythritol (DTE) (fig.3). 20 mM DTE gave nearly complete reversion. Dark incubations with DTE were without effect. Other thiols as 1,2-dimercaptopropanol,  $\beta$ -mercaptoethanol or cysteine can replace DTE. When ADP and  $\text{P}_i$  were also present during the second illumination they prevented the reversal by DTE (not shown).

Careful titrations showed that about 300 nmoles of *o*-iodosobenzoate per mg of chlorophyll reacted with chloroplasts when incubated in the light (table 3). The reaction was prevented by ADP and  $\text{P}_i$  or by uncouplers like  $\text{NH}_4\text{Cl}$  while the energy transfer inhibitor discarine B was ineffective. *o*-Iodosobenzoate did not react with chloroplasts in the dark, in agreement with our previous finding that DTNP only reacted with chloroplast monothiol in the dark [6].

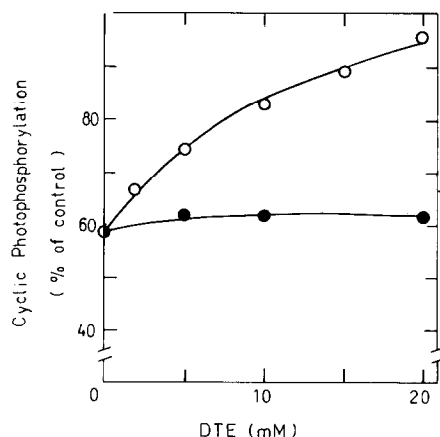


Fig.3. Reversal of *o*-iodosobenzoate inhibition by a second preillumination of chloroplasts with DTE. Chloroplasts were preincubated in the light with 2 mM *o*-iodosobenzoate as described in the text. After turning off the light the stated concentrations of DTE were added and followed by a 2 min period in the dark (● — ●) or in the light (○ — ○). Photophosphorylation was determined as described in the text.

Table 3  
Determinations of *o*-iodosobenzoate reacting with chloroplasts in the light

Additions (mM)	<i>o</i> -Iodosobenzoate disappeared (nmoles/mg chlorophyll)
<i>o</i> -Iodosobenzoate (1)	206
<i>o</i> -Iodosobenzoate (2)	308
<i>o</i> -Iodosobenzoate (4)	316
<i>o</i> -Iodosobenzoate (1), ADP (2), P <sub>i</sub> (2)	48
<i>o</i> -Iodosobenzoate (1), NH <sub>4</sub> Cl (5)	17
<i>o</i> -Iodosobenzoate (1), Discarline B (0.2)	213

Chloroplasts (100  $\mu$ g of chlorophyll) were incubated in the light for 2 min with the additions stated as described in the text. Then, they were centrifuged in the dark for 4 min in an Eppendorf Microcentrifuge. To the supernatant solutions a slight excess of DTE was added and the *o*-iodosobenzoate disappeared was calculated from DTE determination by DTNP as described elsewhere [22]. No *o*-iodosobenzoate reacted with chloroplasts in the dark at any of the concentrations stated.

#### 4. Discussion

All the results described above are in harmony with those obtained with DTNP [6], and are in agreement with other light-dependent inhibitions of photophosphorylation [4,12,13]. These studies take into account that light induces a conformational change in the thylakoid membrane as initially shown by Ryrie and Jagendorf [14,15] and recently confirmed by Kraayenhof and Slater [16].

We have proposed in the preceding paper [6] that the light-dependent inhibition of ATP synthesis and hydrolysis by DTNP may be related to the exposure of vicinal dithiols by a conformational change of CF<sub>1</sub> induced by energization of the thylakoid membrane. The fact that we were able to duplicate with *o*-iodosobenzoate the results observed with DTNP, considering that the former is a selective oxidant reagent for thiol groups confirms our assumption that hidden thiol groups, close enough to be oxidized to disulfide, are uncovered by light. The alternative explanation that light may photoreduce preexisting disulfides [17] is not supported by our results since uncouplers and ADP plus P<sub>i</sub> prevented the *o*-iodosobenzoate or DTNP effects but at the same time they enhanced electron transport. Moreover, reversal of the inhibitory effects of DTNP and *o*-iodosobenzoate was not achieved by a second preillumination alone but required high con-

centrations of DTE (fig.3, and [6]) and this reversion was also prevented by ADP plus P<sub>i</sub>. Similarly, ADP an P<sub>i</sub> prevented the discharge of <sup>3</sup>H from CF<sub>1</sub> by reillumination [15]. Localization of the thiol groups oxidized by DTNP or *o*-iodosobenzoate in or very near to CF<sub>1</sub> is supported by inhibition of the trypsin-activated Ca-ATPase by these reagents (fig.1, and [6]) and by the protection afforded by low concentrations of adenine nucleotides (table 1 and see [18]).

Sequential treatments of chloroplasts in the light with *N*-ethylmaleimide and DTNP or *o*-iodosobenzoate resulted in no additivity of their partial inhibitory effects (unpublished results). These experiments suggest that these reagents reacted with the same thiol groups. At least one of them should be located in a CF<sub>1</sub>  $\gamma$  subunit according to McCarty and Fagan [5]. Since two thiols are required for oxidation to disulfide by DTNP or *o*-iodosobenzoate, the second thiol may belong to the same or to a different CF<sub>1</sub> subunit.

The amount of chloroplasts vicinal dithiols reacting in the light with *o*-iodosobenzoate (table 3) was higher than that reacting with DTNP [6]. When it was expressed per mole of CF<sub>1</sub> according to the chlorophyll to CF<sub>1</sub> ratios calculated by different workers [19,20] the figures resulting are too large to be accounted for only by the CF<sub>1</sub> thiols. The obvious conclusion is that a light-induced conformational change of the thylakoid membrane expose vicinal dithiols in proteins other than

CF<sub>1</sub>. This observation is in agreement with the finding of McCarty and Fagan [5] that there was a light-dependent binding of labeled *N*-ethylmaleimide to several chloroplast proteins. It is surprising that the exposition by light of such a large number of dithiols was completely prevented by uncouplers and specially by ADP plus P<sub>i</sub>.

Attempts to a more precise identification and localization of the dithiols affected by DTNP and *o*-iodosobenzoate are currently being made.

In conclusion our results suggest that the light-dependent oxidation of some vicinal dithiols by two different sulphhydryl reagents 'freezes' CF<sub>1</sub> in a conformational state that partially prevents ATP synthesis and hydrolysis. Although it is unlikely that these dithiols are related to the catalytic site(s) they are required for fully enzymatic activity.

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