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SULPHYDRYL GROUPS IN PHOTOSYNTHETIC ENERGY CONSERVATION

V. LOCALIZATION OF THE NEW DISULFIDE BRIDGES FORMED BY *o*-IODOSOBENZOATE IN COUPLING FACTOR OF SPINACH CHLOROPLASTS

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Summary

1. Chemical modification by *o*-iodosobenzoate of soluble chloroplast coupling factor 1 (CF_1) during heat activation resulted in inhibition of its Ca-ATPase activity and in the formation of two new intrapeptide disulfide bridges as suggested by:

(a) the disappearance of three out of four accessible thiol groups, two from γ and one from a β subunit as a consequence of CF_1 modification by *o*-iodosobenzoate;

(b) the total free sulphydryl groups of CF_1 were reduced from 8 to 4 after modification of CF_1 by *o*-iodosobenzoate. Two groups disappeared from β and two from γ subunits;

(c) a second heating step of CF_1 in the presence of 10 mM dithioerythritol reversed the inhibition of the ATPase and reduced both the newly formed disulfide bridges and those present in native CF_1.

2. Modification of chloroplasts in the light with *o*-iodosobenzoate resulted in the inhibition of photophosphorylation and ATPase. CF_1 isolated and purified from these chloroplasts had its Ca-ATPase activity inhibited and two new disulfide bridges. The total number of free sulphydryl groups was reduced from 8 to 4 and three accessible groups disappeared from β and γ subunits.

Abbreviations: CF_1, chloroplast coupling factor 1; *N*-ethylmaleimide-CF_1, CF_1 with its four accessible sulphydryl groups blocked by *N*-ethylmaleimide.

Introduction

Resolution and reconstitution experiments of spinach chloroplast photophosphorylation has led to the isolation and purification of coupling factor 1 (CF_1^*) the structure and function of which are being throughly studied [1]. CF_1 has a molecular weight of 325 000 [2] and can be separated into five different subunits by dodecyl sulphate polyacrylamide gel electrophoresis [3,4]. The α, β, γ, δ and ϵ subunits have molecular weights of 59 000, 56 000, 37 000, 17 500 and 13 000, respectively [1,4].

The purified CF_1 contains according to Farron and Racker [5] 12 half-cystines per mol, 8 of which are free thiol groups and 4 are involved in 2 disulfide bridges. However, Binder et al. [6] have recently found 14 half-cystines per mol of CF_1.

In CF_1 only two sulphydryl groups are available to sulphydryl reagents [5] but after heat activation of the ATPase two additional sulphydryl groups of the γ subunit become exposed [5,7]. The ATPase activity of CF_1 was not affected by sulphydryl alkylating agents like iodoacetamide or *N*-ethylmaleimide even though they blocked groups that become accessible after heat activation [7].

We have recently shown [8], that sulphydryl reagents like *o*-iodosobenzoate or 2-2′-dithio-bis(5-nitropyridine) only inhibited the heat-activated ATPase of CF_1 when present during the activation. The inhibition was attributed to the oxidation of vicinal dithiols exposed during heat activation. The reversal of inhibition only by a second heating step in the presence of dithioerythritol suggested that the disulfides formed by *o*-iodosobenzoate or 2-2′dithio-bis-(5-nitropyridine) in CF_1 were protected and required a second heating for exposing it again [8—10]. Alkylating agents also inhibited the ATPase of CF_1 when present during heating. Both types of sulphydryl reagents, alkylating and oxidizing were able to inhibit photophosphorylation and related reactions when preincubated in the light with spinach chloroplasts [9—12]. The effect of *o*-iodosobenzoate and 2-2′-dithio-bis-(5-nitropyridine) was attributed to the oxidation of vicinal dithiols of bound CF_1 exposed by a conformational change induced by light. Reversal of the inhibition was achieved by a second illumination in the presence of 20 mM dithioerythritol [10].

In this paper we show that two vicinal dithiols of CF_1 are oxidized by *o*-iodosobenzoate, when chloroplasts are treated with the reagent in the light or when CF_1 is heat-activated in its presence. One of these new disulfide bridges is localized in the γ subunit and the other in a β subunit.

Material and Methods

Materials

Tricine, dithioerythritol, *o*-iodosobenzoate, *N*-ethylmaleimide and nucleotides were obtained from Sigma Chemical Co. All other chemicals were of analytical grade.

The *N*-[^{3}H]ethylmaleimide (specific radioactivity 160 Ci/mol) was obtained from New England Nuclear, 2,5-diphenyloxazole (PPO) and naphthalene from Beckman and 1,4-dioxane from Baker Chemical Co.

Analytical methods

The latent ATPase of CF_1 was activated by heat and assayed as described earlier [8]. Cyclic photophosphorylation [13] Ca-ATPase of chloroplasts [9] protein [14] and chlorophyll concentration [15] were assayed by the published procedures.

Dodecyl suphate gel electrophoresis was performed on a vertical gel apparatus using 10% acrylamide and 0.27% bis-acrylamide in the presence of 0.1% dodecyl sulphate as described by Weber and Osborn [16]. Protein subunits were separated by incubation at 25°C for 2 h in 10 mM sodium phosphate buffer (pH 7) containing 1% dodecyl sulphate.

After electrophoresis, the gels were stained as described [16] or were cut into 1 mm sections according to Matsumura and Noda [17]. With radioactive samples each section was cut in half, one of which was stained with Coomasie brilliant blue at 50°C for 2 h and destained by several washings with a mixture of 7.5% acetic acid and 5% methanol. The fixed dye was extracted by incubation of the gels with 1 ml of 25% pyridine overnight at room temperature [6]. The absorbance at 605 nm of the pyridine extract was measured. The other half of each slice was dissolved in 0.3 ml of H_2O_2 (40 v/v) in sealed scintillation vials for 5 h at 50°C. After cooling, 5 ml of scintillation fluid (5 g of PPO and 100 g of naphthalene in 1 l of 1,4-dioxane) was added and the radioactivity of *N*-[^{3}H]ethylmaleimide was counted in a Beckman LS-233 liquid scintillation counter with an efficiency of 48% determined with an internal standard. This procedure is tiresome but allows an accurate and unequivocal correlation between the localization of the radioactivity label and the stained protein subunits.

Chloroplast isolation

Chloroplasts were isolated from market or greenhouse-grown spinach leaves (*Spinacea oleracea* L) as previously described [14] and resuspended in 250 mM sucrose, 20 mM Tris-HCl (pH 7.8) and 5 mM $MgCl_2$.

CF_1 preparation

Spinach chloroplasts were prepared as described above except that the isolation medium was 400 mM sucrose, 20 mM Tris-HCl at pH 7.8 and 10 mM NaCl. Then the extraction and purification of CF_1 was performed according to Binder et al. [6] except that the washing of DEAE-cellulose in a Buchner funnel was made with 20 mM Tris-SO_4 (pH 7.1), 2 mM EDTA, 1 mM ATP and 100 mM $(NH_4)_2SO_4$ and CF_1 was eluted using the same buffer containing 280 mM $(NH_4)_2SO_4$.

The next purification step was made according to Lien and Racker [18] except that a DEAE-Sepharose CL-6B column (1.25 cm × 25 cm) was used. The purified latent enzyme was stored as an ammonium sulfate suspension at 4°C.

Polyacrylamide gel electrophoresis showed the coupling factor 1 to be at least 95% pure, and had a specific Ca-ATPase activity of 14—20 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ after heat-activation of the enzyme. A molecular weight of $3.25 \cdot 10^5$ [9] was used to obtain the molarity of CF_1.

Preparation of CF_1 derivatives

N-Ethylmaleimide-CF_1: the two sulphydryl groups of native CF_1 and the two sulphydryl groups that become exposed on γ subunit by heat activation of the enzyme were blocked with unlabeled *N*-ethylmaleimide (10 mM) essentially as described by Cantley and Hammes [10], except that the excess of reagent was removed by ultrafiltration-centrifugation [19] in Sephadex G-50 fine columns equilibrated with 20 mM Tricine/2 mM EDTA (pH 8).

Modification of CF_1 with *o*-iodosobenzoate during heat activation of the ATPase was performed in a reaction medium (1 ml) containing 40 mM Tricine/2 mM EDTA (pH 8), 40 mM ATP, 5 mM *o*-iodosobenzoate and 0.5 to 1 mg of CF_1. Heat activation was carried out at 63°C for 4 min. After cooling with running tap water the ATPase activity was assayed. This treatment induced an inhibition of about 50% in the unmasked ATPase [8].

Titrations of sulphydryl groups of CF_1 and CF_1 derivatives

Colorimetric estimation of sulphydryl groups with 2,2'-dithio-bis(5-nitropyridine) was performed according to Grassetti and Murray [20], using a molar extinction coefficient of $1.4 \cdot 10^4$ at 386 nm.

The protein was denatured in buffer 40 mM Tris-HCl (pH 8) containing 2 mM EDTA and 8 M urea for 1 h. In the thiol determination full color develops with 2,2'-dithio-bis-(5-nitropyridine) within 30 min.

Radiochemical titration of sulphydryl groups was performed by incubation of 1 mg of CF_1 with 2 mM *N*-[^{3}H]ethylmaleimide (14Ci/mol) in 1% dodecyl sulphate. The excess reagent was eliminated by ultrafiltration-centrifugation [19] as above, except that the Sephadex G-50 was equilibrated with 10 mM sodium phosphate (pH 7) and 1% dodecyl sulphate. Protein and radioactivity was determined as above.

Results

Disulfide bridge formation in soluble CF_1 by o-iodosobenzoate

We have previously suggested [8] that the inhibitory effect of *o*-iodosobenzoate was the consequence of the formation of new disulfide bridges in CF_1. To check this suggestion the experiments of Table I were carried out. The number of free sulphydryl groups of CF_1 was determined by two different methods: a colorimetric one in the presence of 8 M urea and a radiochemical one in the presence of dodecyl sulphate. With both methods we confirmed that heat-activated CF_1 has 8 sulphydryl groups [5] (Table I, Exp. 1) but when the factor was modified by 5 mM *o*-iodosobenzoate the number of free sulphydryl groups was reduced from 8 to 4 while the Ca-ATPase activity was inhibited by 38%.

CF_1 has two accessible sulphydryl groups [5,7] and two more appear after heat activation. Alkylation of these four groups with *N*-ethylmaleimide reduced the number of total free sulphydryl groups from 8 to 4 as shown in Table I, Expt. 2, but has no effect on the Ca-ATPase activity [7]. Subsequent treatment of the *N*-ethylmaleimide-CF_1 with *o*-iodosobenzoate during a second heating step inhibited the ATPase by 36% and resulted in the disappearance of only two sulphydryl groups. Thus although modification of soluble CF_1 with

TABLE I

EFFECT OF IODOSOBENZOATE ON SULPHYDRYL GROUPS AND ATPase ACTIVITY OF CF_1

Heat activation of CF_1 or *N*-ethylmaleimide-CF_1 (1 mg/ml) was carried out as described in the text in the absence or presence of 5 mM *o*-iodosobenzoate. The Ca-ATPase activity was determined in aliquots of these CF_1 derivatives as described in the text. Total number of free sulphydryl groups was determined by two different methods: A: spectrophotometically by titration of CF_1 derivatives dissociated in 8 M urea with 2,2'-dithio-bis-(5-nitropyridine); radiochemically by labelling of CF_1 derivatives dissociated in dodecyl sulphate with *N*-[^{3}H]ethylmaleimide as described in the text. Numerals in parenthesis indicate inhibition per cent.

Expt.	Treatment of CF_1	Ca-ATPase (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$)	Sulphydryl groups (mol/mol CF_1)	
			A	B
1	CF_1 heat activated	19.4	7.6	7.8
	CF_1 heat activated in the presence of *o*-iodosobenzoate	12 (38)	3.8	4.3
2	*N*-Ethylmaleimide-CF_1, heat activated	20	3.9	—
	N-Ethylmaleimide-CF_1, heat activated in the presence of *o*-iodosobenzoate	12.8 (36)	2.2	—

o-iodosobenzoate resulted in the formation of two new disulfide bridges only one of them is related to the inhibition of the Ca-ATPase in *N*-ethylmaleimide-CF_1.

The two disulfide bridges formed by *o*-iodosobenzoate in CF_1 were intrapeptidic since the mobility of its subunits during dodecylsulphate electrophoresis in the absence of β-mercaptoethanol was exactly the same before and after modification (not shown).

Localization on the new disulfide bridges in soluble CF_1

Table II shows that heating of CF_1 increased the accessible sulphydryl groups from 1.9 to 3.9 and that heating CF_1 with *o*-iodosobenzoate gave the expected inhibition of the Ca-ATPase (37%) and reduced the number of available groups

TABLE II

EFFECT OF HEATING AND *o*-IODOSOBENZOATE ON THE ATPase AND ACCESSIBLE SULPHYDRYL GROUPS OF CF_1

Treatments of CF_1 and determination of ATPase activity were carried out as described in the text. The accessible sulphydryl groups were titrated by incubation of aliquots of CF_1 with 2 mM *N*-[^{3}H]ethylmaleimide (14 Ci/mol) for 1 h. The excess of reagent was removed by ultrafiltration-centrifugation and protein and incorporated radioactivity determined as described in the text.

Treatment of CF_1	ATPase activity (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$)	Sulphydryl groups (mol *N*-[^{3}H]ethylmaleimide/mol CF_1)
None	2.64	1.90
Heat activation	16.50	3.90
Heat activation, *o*-iodosobenzoate	10.40	1.00

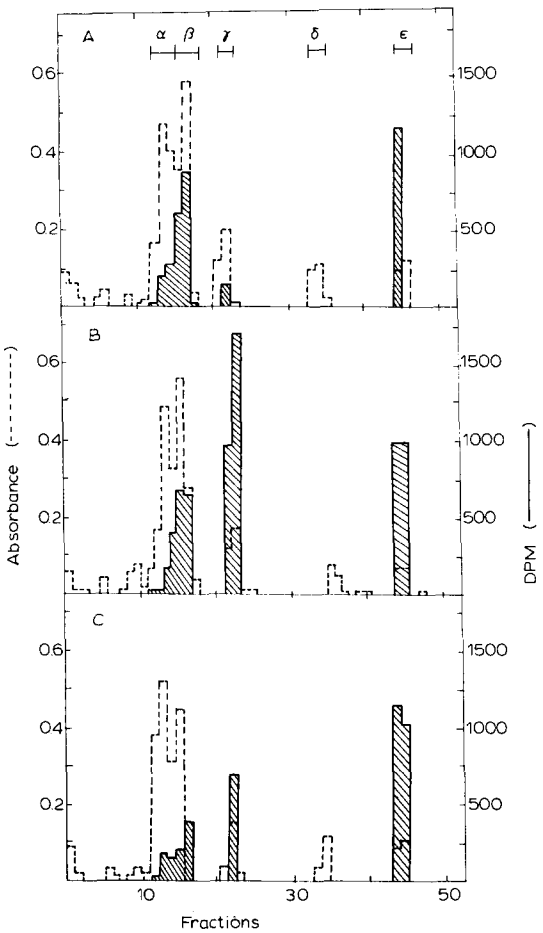

Fig. 1. Subunit localization of the accessible sulphydryl groups of CF_1 affected by *o*-iodosobenzoate. Native CF_1 (Expt. A) was heat-activated in the absence (Expt. B) or in the presence (Expt. C) of 5 mM *o*-iodosobenzoate. The it was treated with *N*-[^{3}H]ethylmaleimide as described in the legend to Table II; The incorporation of *N*-ethylmaleimide was 1.9; 3.9 and 1 mol of *N*-ethylmaleimide per mol of CF_1, for experiments A, B and C, respectively. Dodecyl sulphate electrophoresis was performed in 10% gels as described in the text. Open bars with broken lines, absorbance at 605 nm; hatched bars, radioactivity (dpm).

from 3.9 to 1. The two accessible sulphydryl groups of native CF_1 were found one in β, and the other one in the ϵ subunits (Fig. 1A). Only 4% of the radioactivity was found in the γ subunit. The two sulphydryl groups that appear during heating (Table II) belong to the γ subunit (Fig. 1B) as previously shown [7]. Modification by *o*-iodosobenzoate resulted in the disappearance of 3 sulphydryl groups, two from γ subunit and 1 from a β subunit. However some labeling of the β and γ subunits was observed (Fig. 1C). These results suggest that iodosobenzoate has formed a disulfide bound in a β subunit and another one in γ subunit.

The subunit distribution of free sulphydryl groups in CF_1 heat activated in the absence or presence of *o*-iodosobenzoate was determined in an experiment

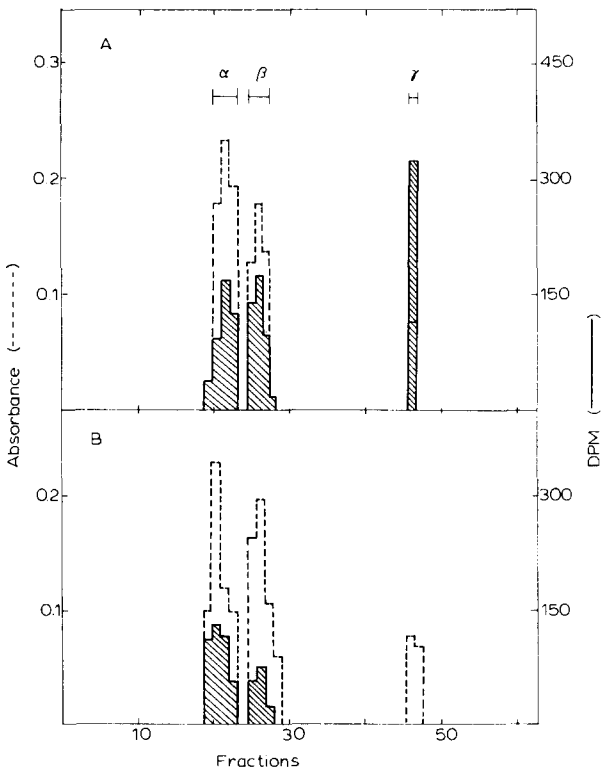

Fig. 2. Subunit localization of free sulphydryl groups affected by *o*-iodosobenzoate. CF_1 was heat activated in the absence (A) or presence (B) of 5 mM *o*-iodosobenzoate. Then it was treated with *N*-[^{3}H]ethylethylmaleimide; 8.2 and 4.2 mol per mol of CF_1 were incorporated, in the experiments A and B, respectively. Dodecyl sulphate and electrophoresis in 12% gels for 17 h was performed as described in the text. Under these conditions complete resolution between α and β subunits was achieved and δ and ε subunits ran off the gel. Open bars with broken lines, absorbance at 605 nm; hatched bars, radioactivity (dpm).

similar to that of Table I, Expt. 1B. Dodecyl suphate polyacrilamide electrophoresis was carried out with 10 and 12% gels, the latter run long enough to resolve α and β subunits. Fig. 2 shows the distribution of the thiol groups in the α, β and γ subunits. In this experiment heat activated CF_1 had 8.2 free sulphydryl groups distributed 1.97 in α, 2.06 in β, 2.12 in γ (as shown in Fig. 2A) and 2.05 in ε subunit (not shown). Heat activation of CF_1 in the presence of 5 mM *o*-iodosobenzoate reduced the free sulphydryl groups to 4.2 per mol of CF_1 and inhibited by 35% its ATPase activity. The groups were found 1.76 in α, 0.62 in β and 1.77 in ε subunits. Fig. 2 shows that the 2 sulphydryl groups from γ subunit and 1.44 groups from a β subunit have disappeared suggesting that iodosobenzoate has formed two new disulfide bonds in β and γ subunits.

Reversal of o-iodosobenzoate effect by dithioerythritol: reduction of disulfide bridges of CF_1

Table III shows that, as previously shown [8], the inhibition of the Ca-ATPase activity of CF_1 by *o*-iodosobenzoate can be reversed by a second heating in the presence of 10 mM dithioerythritol. This reversal of the inhibition

TABLE III

REVERSAL OF *o*-IODOSOBENZOATE EFFECT AND REDUCTION OF DISULPHIDE BRIDGES OF CF_1

Experimental conditions were as described in the text. After each heating steps reagents were removed by ultrafiltration-centrifugation [19].

Additions during heating steps of CF_1		ATPase (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$)	Sulphydryl groups (mol *N*-[^{3}H]ethyl-maleimide/mol CF_1)
First	Second		
None	None	14.6	7.70
o-Iodosobenzoate	None	9.9	4.07
None	Dithioerythritol	19.4	13.01
o-Iodosobenzoate	Dithioerythritol	18.7	12.73

was accompanied with the reduction of all the disulfide bridges of CF_1 i.e. the new disulfide bridges formed by iodosobenzoate and those present in native CF_1 (Table III).

Localization of the effect of o-iodosobenzoate treatment of chloroplasts in the light on bound-CF_1: formation of new disulfide bridges

We have previously shown that preincubation of spinach chloroplasts in the light with 2-2′-dithio-bis-(5-nitropyridine) or *o*-iodosobenzoate resulted in inhibition of photophosphorylation, Mg-ATPase and coupled electron transport [9—10]. This effect was tentatively localized in bound-CF_1 and attributed to the oxidation by the reagents of vicinal dithiols of CF_1 exposed by a light-induced conformational change.

To confirm this hypothesis CF_1 was extracted and purified from control and treated chloroplasts and the number and distribution of sulphydryl groups was determined as above for the soluble CF_1.

TABLE IV

MODIFICATION OF BOUND CF_1 BY LIGHT TREATMENT OF CHLOROPLASTS WITH *o*-IODOSOBENZOATE

Chloroplasts (2 mg chlorophyll/ml) were illuminated for 2 min in the absence or presence of 5 mM *o*-iodosobenzoate and cyclic photophosphorylation and ATPase were determined in aliquots as described [9]. After treatment with *o*-iodosobenzoate the photophosphorylation was decreased from 332 to 132 μmol ATP $\cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$, and the ATPase of chloroplasts from 140 to 91 μmol $P_i \cdot mg^{-1}$ chlorophyll $\cdot h^{-1}$. Then CF_1 was solubilized and purified (DEAE-Sepharose column omitted), the latent ATPase was activated by heat and assayed, and the stoichiometry and subunit distribution of sulphydryl groups were carried out as described in the text. A: total free sulphydryl groups determined radiochemically in dodecyl sulphate; B: accessible sulphydryl groups determined as in Table II. Numeral in parenthesis indicate inhibition per cent.

Treatment of chloroplasts	ATPase of CF_1 (μmol $P_i \cdot min^{-1} \cdot mg^{-1}$)	Sulphydryl groups (mol/mol CF_1)						
		A	B	Subunit distribution (B)				
				α	β	γ	δ	ϵ
Light	13.4	7.73	3.95	0	0.75	2.19	0	1.0
Light, *o*-iodosobenzoate	5.2 (61)	4.28	1.07	0	0.08	0.04	0	0.95

Table IV shows that modification of chloroplasts in the light with *o*-iodosobenzoate gave the expected inhibition of photophosphorylation and of Mg-ATPase. The Ca-ATPase of CF_1 solubilized and purified from the modified thylakoid membranes was also inhibited and the number of its free sulphydryl groups in dodecyl sulphate was decreased from 8 to 4. The number of accessible sulphydryl groups decreased from 4 to 1 and the subunit distribution clearly shows that two groups disappeared from γ subunit and one from β subunit.

Discussion

In this paper we present evidence supporting our suggestion that the light-dependent inhibition of photophosphorylation and related reactions in spinach chloroplasts and the inhibition of Ca-ATPase of soluble CF_1 by *o*-iodosobenzoate [9,10] were the consequence of the oxidation of vicinal dithiol by the reagent. Two new disulfide bonds were formed in thylakoid bound or in soluble CF_1 as shown from the number of accessible and free sulphydryl groups that disappeared after modification (Table II and Figs. 1 and 2). The two new disulfide bonds were intrapapetidic and localized one in a β subunit and the other in the γ subunit.

The vicinal dithiols were hidden from the reagent both in thylakoid bound CF_1 and in soluble CF_1. It is striking that the same hidden dithiols were exposed to *o*-iodosobenzoate by such different methods as a light induced conformational change of the thylakoid bound CF_1 and heating at 63°C of soluble CF_1. Reversion of the inhibitory effects and reduction of the disulfide bonds formed was only obtained when the same procedures were carried out in the presence of dithioerythritol (Table III and ref. 8).

McCarty and coworkers [11,12] have found that incubation of chloroplasts with *N*-ethylmaleimide in the light inhibited photophosphorylation and resulted in the incorporation of *N*-ethylmaleimide into the γ subunit of CF_1. Heat activation of CF_1 in the presence of *N*-ethylmaleimide partially inhibited the ATPase [8]. Sequential treatments of chloroplasts in the light or of soluble CF_1 during heating steps with *o*-iodosobenzoate or 2-2′-dithio-bis-(5-nitropyridine) and *N*-ethylmaleimide resulted in no additivity of their partial inhibitory effects [8—10]. Therefore it is likely that the sulphydryl group of γ subunit blocked by *N*-ethylmaleimide is one of those oxidized by iodosobenzoate or 2-2′-dithio-bis-(5-nitropyridine).

A bifunctional maleimide was recently reported by Weiss and McCarty [21] to uncouple chloroplasts by probably cross-linking sulphydryl groups in the γ subunit of bound CF_1. It would be very interesting to see if the groups involved were the same oxidized by iodosobenzoate which behaves as an energy transfer inhibitor [10].

A vicinal dithiol has recently been found in pig heart mitochondrial F_1 [22].

Several observations made in this work such as the fact that only 1 sulphydryl group of one β subunit (there are 2 β subunits per CF_1 with 2 sulphydryl groups each [6]) is accessible (Fig. 2A) in CF_1 suggest a rather asymmetric positioning of some subunits into the molecular structure of CF_1. This point and an asymmetric redox state of some of the subunits of CF_1 will be discussed in a following paper (manuscript in preparation).

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