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FUNCTIONAL SULFHYDRYL GROUPS OF FERREDOXIN-NADP$^+$ OXIDOREDUCTASE

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Chemical modification of membrane-bound ferredoxin-NADP$^+$ oxidoreductase with oxidants of vicinal dithiols caused inactivation of NADP$^+$ photoreduction, with no effect on the diaphorase activity. Inactivation was partially prevented by ferredoxin and reversed by dithioerythritol. *N*-Ethylmaleimide inhibited both activities, even though with a different kinetic pattern. Inactivation of NADP$^+$ reduction by either *N*-ethylmaleimide or *o*-iodosobenzoate was greater in the light than in the dark. The results suggest the existence of essential sulfhydryl groups related with the ferredoxin site, in addition to those described in the soluble flavoprotein. The role of SH residues in the activity and regulation of membrane bound reductase is discussed.

Introduction

Ferredoxin-NADP$^+$ oxidoreductase (EC 1.18.1.2) participates in the photosynthetic electron flow in algae and higher plants, mediating the electron transfer between ferrodoxin, reduced at the level of Photosystem I, and the two-electron acceptor NADP$^+$ [1,2].

The flavoprotein, which is tightly bound to the outer surface of the thylakoid membrane [3] by van der Waal's forces competing with electrostatic repulsion [4], also shows diaphorase and transhydrogenase [6] activities. The membrane-bound form of the enzyme undergoes a light-dependent transition, as suggested by different lines of evidence: (i) The affinity of the enzyme for its physiological substrates, i.e., ferredoxin and NADP$^+$, was higher in the light than in the dark [7]; (ii) accessibility to specific modifiers was enhanced by illumination of the thylakoids; both effects were prevented by uncouplers [7,8], suggesting that the light-driven ΔpH might induce a conformational change in the membrane-bound enzyme; (iii) experiments carried out with dark-adapted intact chloroplasts revealed a light activation of the electron transport at the level of ferredoxin-NADP$^+$ oxidoreductase prior to any detectable CO_2 fixation [9]. The light modulation of photosynthetic electron transport was also observed in chloroplasts of the green algae *Bryopsis* [10].

In addition to this effect, ferredoxin-NADP$^+$ oxidoreductase also shows a pH-dependent transition of its diaphorase activity, with an apparent pK of about 7 [8]. Both transitions may play a role in the regulation in vivo of the membrane-bound enzyme.

Much effort has been devoted to study the participation of amino acid residues in the catalytic activity of this electron carrier. Chemical modification is an important tool in the determination of the nature and biological function of such residues. For instance, the existence of

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoate); Chl, chlorophyll.

essential lysine [11], arginine [12] and carboxyl groups [13] located at the nucleotide-binding domain of the reductase has been described by means of specific functional group modifiers.

Interestingly, the membrane-bound and soluble ferredoxin-$NADP^+$ oxidoreductase presents different behavior with these reagents. Dansylation experiments carried out with the soluble enzyme revealed the existence of an essential lysine group at the $NADP^+$-binding site [11]. Modification of this residue caused complete inactivation of diaphorase activity both in algae [14] and higher plants [11]. These results could not be reproduced with the membrane-bound flavoprotein [7], suggesting that this is a new aspect of the allotopic properties of this enzyme [7] and that in the membrane-bound form of the enzyme lysine is hidden from dansyl chloride. On the other hand, $NADP^+$ photoreduction was fully inactivated by this reagent [7]. The following features could be observed: (i) the rate of inactivation was greater in the light; (ii) inhibition was almost completely prevented by ferredoxin but not by $NADP^+$; and, (iii) full recovery of activity was attained upon addition of dithioerythritol, the protection afforded by ferredoxin, and the effect of dithioerythritol suggested that the modified residue was related with the ferredoxin-binding site and that it was not a lysine residue. Since the most suitable candidate was a cysteine residue, we decided to investigate the possible involvement of SH groups in ferredoxin-linked reactions of the membrane-bound reductase.

It is known that spinach ferredoxin-$NADP^+$ oxidoreductase contains four cysteines and one disulfide bridge per molecule as revealed by amperometric titration [15]. Incubation of the flavoprotein with mercurials resulted in loss of diaphorase activity, accompanied by irreversible dissociation of the FAD moiety [15]. Other sulfhydryl reagents such as *N*-ethylmaleimide rapidly inactivated the enzyme if preincubated in the presence of NADPH, or another reductant able to reduce the prosthetic group [16]. When incubation with *N*-ethylmaleimide was carried out in the absence of reducing power, a different and less accessible SH group was modified. The pH-mediated transition in diaphorase activity in the soluble reductase was totally prevented by blocking of this second residue [17].

Recently, Shahak et al. [18] reported inactivation of $NADP^+$ photoreduction by mono- and bifunctional maleimides which would involve the membrane-bound form of the reductase.

In the present communication, we report the existence of a vicinal dithiol related to the ferredoxin-binding site, and therefore different from the sulfhydryl group essential for diaphorase activity.

Experimental Procedure

Chloroplast isolation and modification. Chloroplasts were isolated from fresh market spinach leaves (*Spinacea oleracea*) as described previously [19] and suspended in a medium containing 250 mM sucrose, 5 mM $MgCl_2$ and 20 mM Tricine-NaOH, pH 8. Total chlorophyll was determined as described in Ref. 20.

Spinach ferredoxin [21] and ferredoxin-$NADP^+$ oxidoreductase [22] were purified as previously described, and their concentrations determined spectrophotometrically using $\epsilon_{420} = 9.7\ mM^{-1} \cdot cm^{-1}$ for ferredoxin [21] and $\epsilon_{456} = 10.7\ mM^{-1} \cdot cm^{-1}$ for the reductase [23].

Treatment of chloroplasts with SH reagents was carried out in the dark or in the light ($1 \cdot 10^6$ $erg \cdot cm^{-2} \cdot s^{-1}$) at 25°C in a medium (1 ml) containing the same buffer and chloroplasts corresponding to 0.4 mg Chl/ml. Modification was stopped by centrifugation for 2 min at $12000 \times g$ in an Eppendorf microcentrifuge. In some experiments the modification was terminated by dilution (100 times) of the sample in the reaction medium. No significative differences were observed between these two procedures.

Enzyme assays. $NADP^+$ photoreduction [4], diaphorase [24] and cytochrome *c* reductase [25] activities, and the photosynthetic electron transport from water to methyl viologen [26] were determined by previously published procedures.

Incorporation of iodo[^{14}C]acetamide into modified ferredoxin-$NADP^+$ reductase. Samples of the soluble flavoprotein (1 mg/ml) were preincubated at 25°C for 2 h with 10 mM *o*-iodosobenzoate in 10 mM Tricine-NaOH, pH 7.4. After desalting by the filtration-centrifugation procedure [27] aliquots were taken to determine protein concentration and

cytochrome *c* reductase activity and then iodo[^{14}C]acetamide was added to a final concentration of 5 mM, in either the presence or absence of 1% SDS. After 3 h of incubation at room temperature, the samples were filtered twice through Sephadex G-25 according to the method of Penefsky [27] and radioactivity incorporated was determined in an aliquot in a Beckman LS 233 liquid scintillation counter using a mixture of 0.5% 2,5-diphenyloxazole and 10% naphthalene in dioxane as scintillation cocktail.

Results

Effect of sulfhydryl group reagents on membrane-bound ferredoxin-NADP$^+$ oxidoreductase

The role of sulfhydryl groups in ferredoxin-NADP$^+$ oxidoreductase was studied by using different specific reagents. Table I shows that oxidants of vicinal dithiols such as diamide, GSSG and iodosobenzoate inactivated NADP$^+$ photoreduction without impairing the diaphorase activity of the chloroplasts. The alkylating reagent iodoacetamide (Table I, Expt. 3) presented the same behavior, at variance with *N*-ethylmaleimide which inhibited both activities. The inactivation of NADP$^+$ photoreduction by iodosobenzoate was partially protected by ferredoxin (Table I, Expt. 7) and was enhanced by light (Table I, Expt. 13). Addition of 100 mM dithioerythritol to thylakoids previously modified by oxidizing reagents totally restored the enzymatic activity (Table I, Expts. 8–10). Full recovery of activity was attained in less than 2 min (data not shown). Photoreduction of the artificial electron acceptor methyl viologen was not affected by any of these reagents, either in the dark or in the light (not shown), indicating that the site of inactivation should be found at the level of ferredoxin-NADP$^+$ oxidoreductase. These results suggest that the oxidation of vicinal dithiols, presumably located at or in the vicinity of the ferredoxin-binding domain of ferredoxin-NADP$^+$ oxidoreductase, was responsible for the inhibition.

The kinetics of inactivation by *N*-ethylmaleimide of the diaphorase activity are shown in Fig. 1. Biphasic behavior was detected, with an initial fast phase up to 35% inhibition followed by a much slower phase. There was no effect of light.

TABLE I

EFFECT OF SEVERAL SULFHYDRYL REAGENTS ON MEMBRANE-BOUND FERREDOXIN-NADP$^+$ OXIDOREDUCTASE

Spinach chloroplasts were pretreated with the reagents stated for 60 min as described in the text either in the dark or in the light. Phenazine methosulfate (33 μM) was present during the incubation in the light. n.d., not determined; diamide, diazenedicarboxylic acid bis(*N*,*N*-dimethylamide).

Expt. No.	Pretreatment of thylakoids	NADP$^+$ photoreduction (μmol NADPH/h per mg Chl)	Diaphorase (μmol $K_3Fe(CN)_6$/h per mg Chl)
In the dark			
1	None	132	410
2	*N*-Ethylmaleimide (10 mM)	51	220
3	Iodoacetamide (5 mM)	83	380
4	Diamide (10 mM)	67	402
5	GSSG (50 mM)	69	398
6	Iodosobenzoate (10 mM)	46	390
7	Iodosobenzoate (10 mM) + ferredoxin (50 μM)	106	nd
8	As for Expt. 4, then dithioerythritol (100 mM)	130	nd
9	As for Expt. 5, then dithioerythritol (100 mM)	129	nd
10	As for Expt. 6, then dithioerythritol (100 mM)	134	nd
In the light			
11	Control	92.7	380
12	*N*-Ethylmaleimide (10 mM)	0	215
13	Iodosobenzoate (10 mM)	17	376

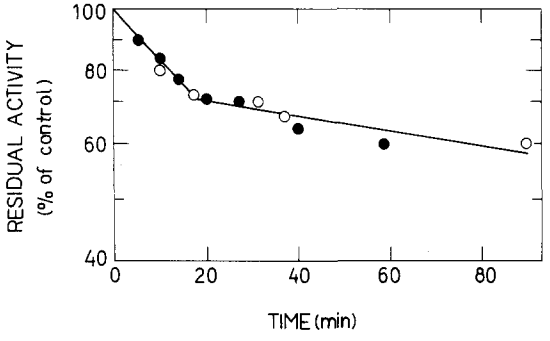

Fig. 1. Inactivation of diaphorase activity of membrane-bound ferredoxin-NADP$^+$ oxidoreductase by *N*-ethylmaleimide. Spinach chloroplasts were preincubated in the light ($1 \cdot 10^6$ erg·cm^{-2}·s^{-1}) in the presence of 33 μM phenazine methosulfate (○——○) or in the dark (●——●) with 10 mM *N*-ethylmaleimide as described in the text. Then diaphorase activity was measured.

Fig. 2 shows that inactivation of NADP$^+$ photoreduction activity followed pseudo-first-order kinetics. When modification was carried out in the light (in the presence of phenazine methosulfate) the inactivation was twice as rapid. The electron flow from water to methyl viologen was not inhibited (Fig. 2). These results suggest that a sulfhydryl group different from the vicinal dithiol involved in NADP$^+$ photoreduction may be related with the inhibition of the diaphorase activity.

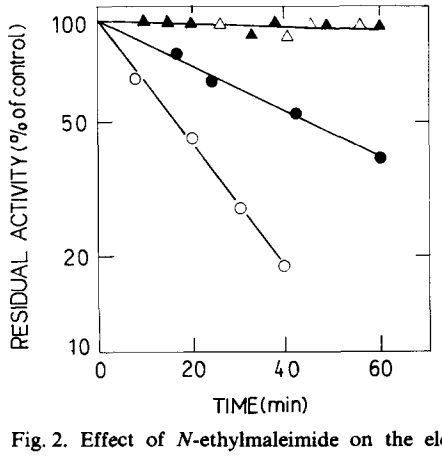

Fig. 2. Effect of *N*-ethylmaleimide on the electron-transport reactions of spinach thylakoids. Methyl viologen (△, ▲) or NADP$^+$ photoreduction (○, ●) was measured in chloroplasts preincubated in the dark (closed symbols) or in the light (open symbols) with *N*-ethylmaleimide as in Fig. 1.

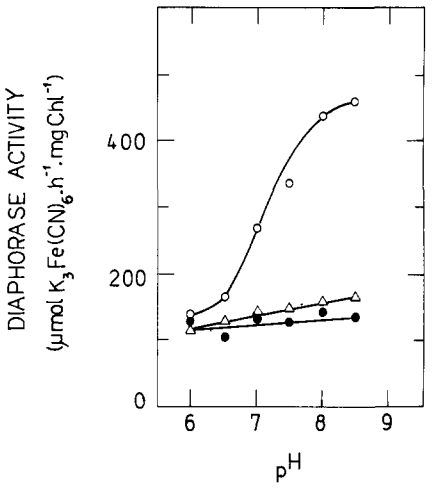

Fig. 3. pH profile for diaphorase activity of spinach thylakoids. Diaphorase activity was measured at the stated pH in a mixture of buffers containing 20 mM each of Mops, Mes and Tricine-NaOH. (○——○) Control chloroplasts were assayed in a medium supplemented with 5 mM $MgCl_2$ and 250 mM sucrose; (●——●) sucrose was replaced by 0.7 M NaCl in the assay medium; (△——△) chloroplasts were pretreated with 10 mM *N*-ethylmaleimide for 2 h prior to assay.

In order to check this assumption, the diaphorase activity of thylakoids modified with *N*-ethylmaleimide was assayed at different pH values. The results are summarized in Fig. 3. As can be seen, the modified enzyme was unable to undergo the pH-driven transition. High ionic strength mimics the effect of *N*-ethylmaleimide (Fig. 3). Our results agree with those found by Davis and San Pietro [17] with the soluble enzyme, although higher concentrations of NaCl were added in order to obtain comparable effects, suggesting that interaction of the enzyme with the membrane may partially protect the membrane-bound enzyme against the effects of salt concentration.

Effect of iodosobenzoate on the purified reductase

Diaphorase activity of purified, soluble ferredoxin-NADP$^+$ oxidoreductase was not affected by prolonged incubation of the flavoprotein with *o*-iodosobenzoate. Nevertheless, inhibition of ferredoxin-linked cytochrome *c* reductase activity was observed (Table II) even though at a smaller rate than that reported for NADP$^+$ photoreduction in Table I.

TABLE II

INCORPORATION OF IODO[^{14}C]ACETAMIDE INTO PURIFIED FERREDOXIN-NADP$^+$ OXIDOREDUCTASE

Experimental conditions are described in the text. A, native enzyme; B, enzyme treated with SDS; C, enzyme treated with SDS and 50 mM dithioerythritol for 30 min before labeling with iodo[^{14}C]acetamide. Value between parentheses indicates per cent of residual activity.

Pretreatment	Cytochrome *c* reductase (μmol cytochrome *c*/min per mg)	Sulfhydryl groups (mol iodo[^{14}C]acetamide/mol enzyme)		
		A	B	C
None	77.8	1.3	3.7	6.5
Iodosobenzoate (10 mM)	32.4 (42)	1.0	1.9	–

In view of the partial protection afforded by ferredoxin against inactivation of NADP$^+$ photoreduction by iodosobenzoate, it was of interest to study the ability of the modified soluble enzyme to interact with the iron-sulfur protein. Ferredoxin-NADP$^+$ oxidoreductase shows a difference spectrum upon complex formation with ferredoxin [28–32]. In Fig. 4 the difference spectra obtained with the native and modified enzyme are compared. It shows that the flavoprotein previously oxidized by iodosobenzoate was unable to complex with ferredoxin.

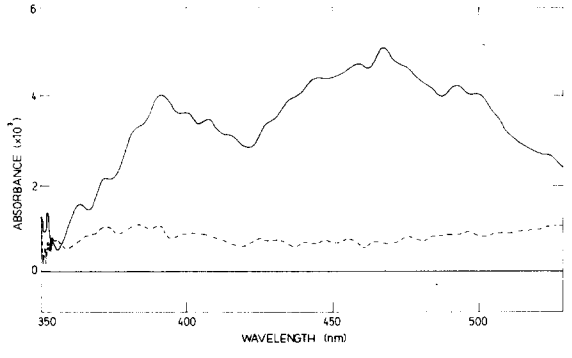

Fig. 4. Complex formation between ferredoxin and native or modified soluble ferredoxin-NADP$^+$ oxidoreductase. Difference spectra between physical and optical mixtures of ferredoxin (19 μM) and soluble enzyme (11 μM) in 10 mM Tricine-NaOH, pH 7.4, were recorded using tandem cell cuvettes in an Aminco DW 2a spectrophotometer equipped with a MIDAN T Microprocessor Data Analyzer. Modification with *o*-iodosobenzoate (10 mM) of the enzyme was carried out at 25°C for 2 h in 10 mM Tricine-NaOH, pH 7.4. Samples were filtered twice through Sephadex G-25 [25] prior to any spectral measurement. (————) Difference spectrum with native enzyme; (------) difference spectrum with modified enzyme.

In order to determine the amount of SH groups affected by iodosobenzoate, radiochemical titration with the alkylating reagent iodo[^{14}C]acetamide was carried out. Using this reagent in the presence of SDS, a total of 3.7 sulfhydryl groups/mol of ferredoxin-NADP$^+$ oxidoreductase was found (Table II), in good agreement with previous reports [15,33]. This value decreased to 1.9 in the modified enzyme titrated under the same conditions (Table II), indicating that the formation of a single disulfide bond should be responsible for the alteration of kinetic properties of the flavoprotein and of the complex formation with ferredoxin. When titration was carried out with the native enzyme, a maximum value of 1.3 iodo[^{14}C]-acetamide/FAD was reached, even after several hours of incubation, without loss of diaphorase activity, indicating a certain degree of inaccessibility of some of the cysteines to iodoacetamide. A similar result was reported earlier with DTNB [34]. Complete reduction of the denatured enzyme resulted in an apparent incorporation of 6,5 mol iodo[^{14}C]acetamide/mol enzyme in accordance with previous results on amino acid composition [33] and amperometric titration [15].

Discussion

Inactivation of NADP$^+$ photoreduction by reagents that oxidize vicinal dithiols was enhanced by illumination of the thylakoids during modification. These reagents have no effect on the diaphorase activity of the enzyme. Complete oxidation of a vicinal dithiol in the purified enzyme was attained by iodosobenzoate as judged by radiochemical

titration with iodo[^{14}C]acetamide (Table II).

The proximal cysteines may be at or near the ferredoxin-binding site of the enzyme, as suggested by the partial protection afforded by ferredoxin against modification with *o*-iodosobenzoate. Moreover, complex formation between both enzymes was suppressed by the treatment.

Inactivation of $NADP^+$ photoreduction by *N*-ethylmaleimide may be distinguished from inactivation of diaphorase activity by: (i) different kinetic patterns of inactivations and (ii) only inactivation of the former was enhanced by light. These results suggest that *N*-ethylmaleimide affects at least two different sulfhydryl groups. One of them may belong to the vicinal dithiol oxidized by iodosobenzoate, may account for the inhibition of $NADP^+$ photoreduction and becomes more accessible during the energy-driven conformational change of ferredoxin-$NADP^+$ oxidoreductase [7,8].

In conclusion, modification of any of the four cysteines [15] found in ferredoxin-$NADP^+$ oxidoreductase affects the catalytic activities of the flavoprotein since: (a) An SH group participates in the attachment of FAD [34]; (b) A structural cysteine is related with the pH-dependent activation of diaphorase activity (Ref. 17 and Fig. 3) and reacts slowly with *N*-ethylmaleimide either in the soluble or membrane-bound form of the enzyme; (c) There is a vicinal dithiol at the ferredoxin site which becomes more exposed to modifiers upon illumination of the thylakoid membranes.

Probably dansyl chloride modified one or both of the SH groups of this vicinal dithiol [8].

Two different types of conformational movements were described in the membrane-bound enzyme; a light-driven one, mediated by the formation of a transmembrane proton gradient [7], and a pH-dependent transition resulting in activation of the diaphorase activity [8]. The possible involvement of these phenomena in the regulation of the enzyme in vivo was previously discussed [8]. The data presented here suggest the existence of two distinct populations of cysteines related with these conformational changes: a sulfhydryl group essential for the pH-dependent transition, and a vicinal dithiol that becomes more accessible to oxidants during the light-driven structural change of the membrane-bound enzyme.

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