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Chemical cross-linking of ferredoxin to spinach thylakoids

Evidence for two independent binding sites of ferredoxin to the membrane

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Ferredoxin has been chemically cross-linked to thylakoids by using *N*-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The membranes thus treated became able to photoreduce cytochrome *c* and to catalyze the NADPH-cytochrome *c* reductase reaction without adding exogenous ferredoxin. Preincubation of thylakoids with an antibody against ferredoxin-NADP$^+$ reductase before carbodiimide treatment or removal of the reductase by mild trypsin treatment after the cross-linking reaction did not alter the cytochrome *c* photoreduction activity of the treated membranes. Two independent binding sites of ferredoxin to thylakoids are thus inferred: one site is shown to be the membrane-bound reductase, the second is suggested to be at the level of the photosystem I complex.

Ferredoxin; Thylakoid; Ferredoxin-NADP$^+$ reductase; Photosystem I; Photosynthesis

1. INTRODUCTION

Ferredoxin, a low-M_r iron-sulfur protein, is the intermediate carrier in the electron flow from the photosystem I complex to ferredoxin-NADP$^+$ reductase (EC 1.18.1.2) in the chloroplast electron-transport chain. Formation of a covalent complex between ferredoxin and the reductase has been achieved by treating the purified proteins with a soluble carbodiimide (EDC) [1]. We explored the possibility of cross-linking ferredoxin to the membrane-bound flavoprotein in order to support our earlier hypothesis that ferredoxin could bridge photosystem I and the reductase, thus providing a continuous channel for the electron flow in the chloroplast chain [1,2]. Alternatively, ferredoxin, which is considered a soluble protein, could shuttle between its membrane-bound partners. In both cases, two sites of interaction of Fd with thylakoids are implied, as recently reported [3]; yet to discriminate between these two hypotheses it is important to demonstrate whether these sites are independent. Here, evidence is given for the existence of two independent binding sites of ferredoxin to the membrane, through cross-linking of the protein to thylakoids by EDC.

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Abbreviations: EDC, *N*-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Fd, ferredoxin; FNR, ferredoxin-NADP$^+$ reductase; cyt., cytochrome

2. MATERIALS AND METHODS

NADP$^+$, horse heart cyt. *c*, EDC, trypsin and trypsin inhibitor were obtained from Sigma. Ferredoxin was purified from spinach as in [4]. Thylakoid preparation: chloroplasts were obtained by sedimentation at 1500 × *g* for 5 min of spinach leaf homogenates in 25 mM K-phosphate, pH 7, containing 0.4 M sucrose and 10 mM NaCl (isotonic solution); the pellet was resuspended in

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hypotonic solution; after 5 min, membranes were either sedimented as above and resuspended in the isotonic solution (washed thylakoids) or used as such after addition of 2 vols of the isotonic solution. Incubation of thylakoids with EDC: thylakoids (0.4–0.6 mg Chl/ml) were treated with 5 mM EDC at 25°C for 15 min in the isotonic solution containing 2 mM $MgCl_2$ and in the presence or absence of 4 μM ferredoxin. The carbodiimide reaction was quenched by adding CH_3COONH_4 to 0.1 M final concentration, followed by a 5-fold dilution with ice-cold isotonic solution. After centrifugation at 1500 × *g* for 5 min, pellets were resuspended in the isotonic solution. A control was always run under the same conditions in the presence of Fd but without EDC. Chlorophyll was determined as in [5]. $NADP^+$ and cyt. *c* photoreduction, and diaphorase and NADPH-cyt. *c* reductase activities were measured as reported [1]. All kinetic measurements were performed on a Cary 219 spectrophotometer.

3. RESULTS AND DISCUSSION

Washed thylakoids were incubated with 5 mM EDC at 25°C for 15 min in the presence or absence of purified ferredoxin. Table 1 lists the effects of such treatments on various activities of thylakoids after removal of excess reagents. Clearly, thylakoids treated with EDC in the presence of Fd acquired the ability to photoreduce cyt. *c* and to catalyze the NADPH-cyt. *c* reductase reaction, without adding exogenous Fd. The extent of Fd independence of these activities was a function of Fd concentration as well as of chlorophyll concentration during EDC incubation (see tables 2 and 3). The endogenous cyt. *c* photoreduction activity of the controls was shown not to be catalyzed by Fd [6]; accordingly, it could be markedly reduced by superoxide dismutase and thus, it was subtracted from all values obtained. On the other hand, the increase in endogenous rates in samples treated with EDC in the absence of added Fd could be due to cross-linking of a small amount of Fd, which is still present in washed thylakoids as shown by the Ouchterlony technique using an anti-Fd antibody. Subsequently, washing of thylakoids was omitted in those cases where removal of endogenous ferredoxin was not required, since it determined a marked decrease (30–50%) of thylakoid activities following the 25°C incubation. The ability of treated thylakoids to catalyze the NADPH-cyt. *c* reductase reaction without addition of exogenous Fd could be explained as arising from the EDC cross-linking of Fd to the membrane-bound reductase, based on previous studies with the purified proteins [1]. The cyt. *c* photoreduction activity which became Fd-independent could also be depicted as involving the Fd moiety of the covalent complex between the two proteins; the $NADP^+$ photoreduction was expected to involve a similar complex: instead, treated thylakoids were unable

Table 1

Effects of EDC incubation on ferredoxin dependence of thylakoids' various activities

Incubation conditions	$NADP^+$ photoreduction		Cytochrome *c* photoreduction		NADPH-cytochrome *c* reductase activity	
	− Fd	+ Fd	− Fd	+ Fd	− Fd	+ Fd
Control	0	100[a]	–	100[b]	1	100[c]
5 mM EDC	0	80	7	96	3	90
5 mM EDC, 4 μM Fd	0	52	54	95	22	65

[a] 100 = 280 μequiv. · mg^{-1} Chl · h^{-1}

[b] 100 = 357 μequiv. · mg^{-1} Chl · h^{-1} (the endogenous rate has been subtracted from all the values shown)

[c] 100 = 74 μequiv. · mg^{-1} Chl · h^{-1}

Values represent the mean of several experiments

to photoreduce $NADP^+$ unless exogenous Fd was added and those treated in the presence of Fd showed a marked inhibition.

To clear this discrepancy, we attempted to prevent Fd binding to the reductase by complexing the enzyme with its antibody. It was shown earlier [7] that the antireductase antibody does not inhibit cyt. *c* photoreduction or other chloroplast FNR-independent reactions. The anti-FNR antibody was added to thylakoids to inhibit fully $NADP^+$ photoreduction before EDC incubation. Table 2 shows that the acquired capacity by thylakoids to photoreduce cyt. *c* remained nearly irrespective of the presence or absence of the antibody. Thus, the antireductase antibody did not prevent the Fd binding to the membrane as measured by cyt. *c* photoreduction. It seems rather unlikely that binding of Fd to the membrane-bound reductase would not be impaired by the anti-FNR antibody, due at least to steric hindrance; however, direct evidence is lacking because of antibody inhibition of the reductase activities.

Another approach to the problem was to remove selectively the reductase from the membrane, after

Table 2

Effects of antireductase antibody addition during the EDC incubation of thylakoids

Incubation conditions	$NADP^+$ photoreduction + Fd	Cytochrome *c* photoreduction[a]		NADPH-cyt. *c* reductase activity	
		− Fd	+ Fd	− Fd	+ Fd
Control	100	–	100	1	100
5 mM EDC	28	70	100	31	46
Anti-FNR antibody + 5 mM EDC	0	64	90	0	0

[a] The endogenous rate has been subtracted from all the values shown

Thylakoids (0.28 mg Chl/ml) were treated with an anti-FNR antibody to inhibit fully $NADP^+$ photoreduction and then incubated with EDC as described in section 2

Table 3

Trypsin treatment of thylakoids after EDC incubation

Conditions	$NADP^+$ photoreduction + Fd	Cytochrome *c* photoreduction[a]		NADPH-cyt. *c* reductase activity (μequiv. $\cdot mg^{-1}$ Chl $\cdot min^{-1}$)			
		− Fd	+ Fd	Pellet		Supernatant	
				− Fd	+ Fd	− Fd	+ Fd
Control	100	–	100	0	0.876	0	0.247
+ trypsin, 60 s	53	–	98	0	0.265	0	1.390
5 mM EDC	51	55	100	0.309	0.573	0.049	0.132
+ trypsin, 60 s	32	50	95	0.090	0.227	0.343	0.885

[a] The endogenous rate has been subtracted from all the values shown

Thylakoids (0.8 mg Chl/ml) were incubated with EDC in the presence of 4 μM Fd as described in section 2. After resuspension in the isotonic solution at pH 8, the membranes were kept at 25°C for 2 min and then incubated with trypsin (1 : 25 (w/w) to chlorophyll); to quench the reaction a 10-fold excess of soybean trypsin inhibitor was added [8]. An aliquot of the samples was immediately centrifuged at 12000 × *g* for 5 min; NADPH-cyt. *c* reductase activity was measured on the pellet resuspended in isotonic solution and on the supernatant. The values represent the mean of 5 experiments

achievement of Fd binding to thylakoids. Mild trypsin treatment [8] was used. The results are summarized in table 3. In such experiments, a decrease between 50 and 70% of FNR-dependent activities was obtained, whereas the cyt. *c* photoreduction activity remained virtually unchanged, in both native and EDC-treated thylakoids. FNR and FNR-Fd (cross-linked) were actually released in the supernatant after sedimentation of the trypsin-treated thylakoids as shown by determination of NADPH-cyt. *c* reductase activity in both the presence and absence of Fd; under our conditions, an increase in NADPH-cyt. *c* reductase activity following trypsin treatment was observed.

These results suggest that there are two independent sites for Fd binding: one endowed with cyt. *c* photoreduction activity, the other being involved in NADPH-cyt. *c* reductase activity. Fd thus appeared to be covalently bound to thylakoids in two independent locations simultaneously. One site was shown to be the membrane-bound reductase by trypsin removal of the cross-linked complex FNR-Fd; the other site is suggested to be a polypeptide of the photosystem I complex, which is the electron donor to Fd in the electron-transport chain, because of the cyt. *c* photoreduction activity acquired by thylakoids after cross-linking of Fd. In this hypothesis, the lack of the $NADP^+$ photoreduction activity (assayed in the absence of added Fd) by treated membranes and the only partial restoration of such activity by addition of exogenous Fd, could be interpreted as due not just to general damage induced by the EDC treatment, but to a specific blocking of the binding sites for Fd by the cross-linked protein. Studies are in progress to verify the hypothesis put forward.

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