PHOTOSYNTHETIC ELECTRON TRANSPORT BY ZWITTERGENT-EXTRACTED CHLOROPLASTS REQUIRES SOLUBILIZED PLASTOCYANIN

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1. Introduction

Evidence from numerous laboratories had demonstrated that light plays a regulatory role in photosynthesis through the modulation of key enzymes [1–3]. One system that has been proposed to account for light-dependent enzyme modulation involves membrane-bound cyst(e)ine-containing proteins, designated 'light effect mediators' or 'LEMs'—components proposed to occur in the oxidized (disulfide) state in the dark and in the reduced (sulfhydryl) state in the light [2,4]. It is visualized that chloroplasts contain two LEMs (both on the acceptor side of photosystem I) that, following reduction by non-cyclic electron transport, modulate enzymes of the stroma.

One of the proposed LEM components can be solubilized from peas by extracting chloroplast membranes with a Zwittergent detergent [5,6]. In the presence of an artificial electron donor, ascorbate/2,6-dichlorophenolindophenol (DCPIP), the Zwittergent-treated chloroplast membranes promoted the photoregulation of NADP-malate dehydrogenase of the stroma. Photoregulation in this system was dependent on the solubilized protein fraction that, based on SDS gel electrophoresis, contained several protein components.

In experiments designed to determine the effect of Zwittergent extraction on enzyme photoregulation by systems under investigation in our laboratory (i.e., the ferredoxin/thioredoxin and ferralterin systems [3,7]) applied the Zwittergent reagent to chloroplast membranes isolated from both spinach and peas. The protein fraction solubilized by the Zwittergent was required by the extracted parent membranes from both sources for the photoreduction of the electron acceptors tested (i.e., ferredoxin, methyl viologen, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) when ascorbate/DCPIP served as electron donor. Here, we summarize these findings and present evidence that the Zwittergent-solubilized component required for electron transport is plastocyanin.

Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; EPR, electron paramagnetic spin resonance

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2. Materials and methods

2.1. *Plants*

Spinach (*Spinacea oleracea*) leaves were obtained from either greenhouse grown plants (var. Hipack, Asgrow Seed Co., Tracy CA) [8] or were purchased from a local market. No differences were noted with the chloroplasts prepared from either of these source materials. Peas (*Pisum sativum* var. Progress no. 9, Ferry-Morse Seed Co., Mt View CA) were germinated and grown in open air in vermiculite under normal day/night conditions.

2.2. *Chemicals*

Chemicals were obtained from commercial sources and were of the highest quality available.

2.3. *Preparative procedures*

Chloroplasts were isolated from 240 g spinach or pea leaves by blending in 600 ml 'blending solution' (50 mM tricine–HCl (pH 7.6) containing 375 mM sucrose, 2 mM $MgCl_2$, 2 mM EDTA-Na, 2 mM sodium ascorbate) [6]. The homogenate was filtered through

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4 layers of cheese cloth and centrifuged 1 min at 3000 × *g*. The supernatant fraction was discarded; the pellet, containing intact chloroplasts, was washed once by resuspension in 120 ml of blending solution and collected by a second centrifugation (1 min at 3000 × *g*).

The washed intact chloroplasts were ruptured, washed and extracted with detergent as in [5,6]. The pellet from the second centrifugation step, containing the once-washed intact chloroplasts from above, was resuspended in 30 ml 'lysing buffer' (50 mM tricine (pH 7.6) 1 mM EDTA-Na, 2 mM $MgCl_2$, 10 mM KCl) and then centrifuged 1 min at 12 000 × *g*. The supernatant (stromal) solution was discarded and the pelleted thylakoid membranes were washed by resuspension in 120 ml lysing buffer, stirred for 10 min at 4°C, and then collected by centrifugation (1 min, 12 000 × *g*).

The washed thylakoid membranes were resuspended in the lysing buffer to 2 mg chlorophyll/ml; an equal volume of 10 mM detergent was then added to give final respective chlorophyll and detergent concentrations of 1 mg/ml and 5 mM. The detergent used was Zwittergent, critical micellar concentration 0.0012%, M_r 391.6 obtained from Cal Biochem (Los Angeles CA). Following detergent addition, the membranes were extracted by stirring for 2 min at 20°C and then collected by centrifugation (30 min, 48 000 × *g*). The green supernatant solution (Zwittergent extract) containing the solubilized proteins was further treated as described below, and the pellet, containing the extracted thylakoid membranes, was washed 3 times by resuspension (100 ml lysing buffer)/centrifugation (10 min, 48 000 × *g*) steps.

For most experiments, freshly prepared membranes were used. In some cases (e.g., determination of column profiles) membranes that had been stored at −20°C were also effectively employed. We observed that photosystem I activity associated with the extracted membranes was stabilized by rapid (liquid nitrogen) freezing in lysing buffer supplemented with 1% bovine serum albumin and 10% glycerol. The membranes stored in this manner were usually stable for ~1 month at −20°C.

The Zwittergent extract obtained above was clarified by high-speed centrifugation (2 h, 270 000 × *g*) and concentrated by overnight dialysis against solid sucrose (at 4°C). Dialysis tubing with an M_r-cutoff of 6000–8000 (Spectrum Medical Industries, Los Angeles CA) was used routinely at this step. The concentrated extract was dialyzed 24 h against 2 l lysing buffer and then concentrated to 10% of its original volume by a second sucrose dialysis. The concentrated Zwittergent extract was then fractionated by filtration on a 1.5 × 75 cm Sephadex G-75 column that had been equilibrated with the lysing buffer. The fractions obtained were routinely monitored for A_{280} and for activity in the NADP photoreduction assay (cf. fig.1).

Published procedures were used for the purification from spinach leaves of ferredoxin [9], ferredoxin-NADP reductase [10] and plastocyanin [11].

2.4. *Analytical procedures*

Chlorophyll was determined according to [12]. Electrophoresis in SDS was done as in [13]. Protein was routinely analyzed by determining A_{280}. Absorption spectra were measured with a Cary 219 spectrophotometer. Electron paramagnetic spin resonance (EPR) spectra were measured as in [14].

3. Results and discussion

3.1. *Purification and properties of the Zwittergent factor*

In initial exploratory experiments, we determined the effect of Zwittergent extraction on the ascorbate/DCPIP-linked photoreduction of NADP, and we consistently observed a requirement by the treated membranes for the solubilized fraction (not shown). In an effort to determine the nature of the component solubilized by the Zwittergent, we attempted to fractionate the solubilized extract by filtration on a Sephadex G-75 column. As shown in fig.1 for a spinach preparation, (the data presented below will pertain to spinach unless indicated otherwise), the component required for NADP photoreduction, designated the Zwittergent factor, was eluted from the column as a small peak, absorbing at 280 nm, after the bulk of the applied protein. Also present were two peaks of ferredoxin-NADP reductase activity, presumably corresponding to the dimer and monomer forms of the enzyme [15], that were solubilized by the detergent. Although consistently present, the amount of ferredoxin-NADP reductase released by the detergent was relatively limited based on the fact that photoreduction of NADP by the extracted membranes proceeded maximally with Zwittergent factor added in the absence of the reductase. Nevertheless, small

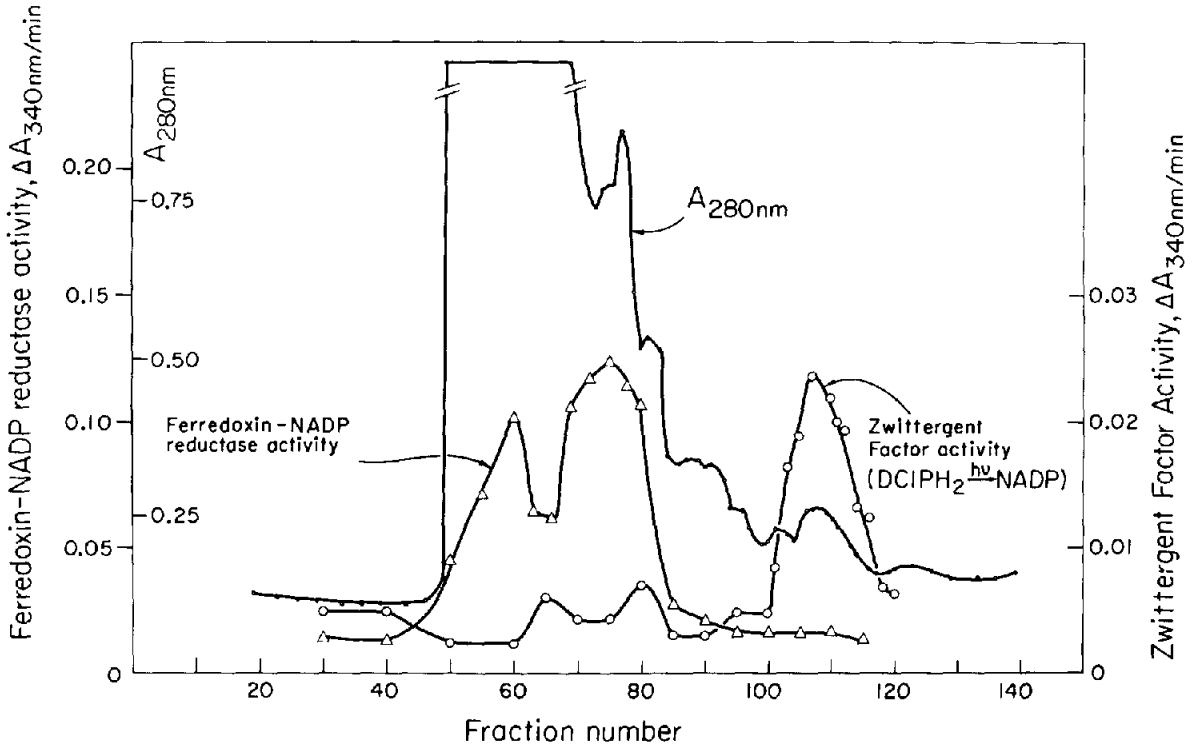

Fig.1. Sephadex G-75 column profile of supernatant fraction of Zwittergent-treated spinach chloroplast membranes. For assaying Zwittergent factor, the reaction mixture contained (in 0.5 ml final vol.) Zwittergent-extracted chloroplast thylakoid membranes equivalent to 80 μg; spinach ferredoxin, 22 μg; spinach ferredoxin-NADP reductase, 2 μg; and the following (in μmol); tricine–HCl buffer (pH 7.7) 50; NH_4Cl (2.5); $MgSO_4$ (5); NADP (1); sodium ascorbate (5); DCPIP (0.05). For assaying ferredoxin-NADP reductase, the same reaction mixture was used except that chloroplast membranes were replaced by *Nostoc muscorum* membrane fragments (C-144) [17] and ferredoxin-NADP reductase was omitted. Photoreduction of NADP was measured at 340 nm in 0.2 cm cuvettes as in [18].

amounts of the reductase were routinely added to the assay mixture as a precautionary measure (cf. fig.1).

Several lines of evidence indicated that the required component solubilized by the Zwittergent was a protein. The active peak from the Sephadex G-75 column, which showed a single Coomassie blue-staining band in SDS-gel electrophoresis, was sensitive both to trypsin digestion and to heat (5 min at 85°C destroyed 87% of the original activity determined as in table 1). The peak fractions also showed an absorption peak in the ultraviolet at 280 nm with no significant absorption in the visible region.

3.2. *Activity of the Zwittergent factor in different chloroplast reactions*

The purified Zwittergent factor was found to be required for the photoreduction not only of ferredoxin/NADP, but of other electron acceptors as well,

Table 1
Equivalence of Zwittergent factor and plastocyanin in photoreduction of NADP by extracted spinach chloroplast membranes

	NADP reduced (μmol/mg chl. h)
Extracted membranes	0.2
+ 55 μg factor	2.5
+ 52 μg plastocyanin	2.9
+ 55 μg factor + 52 μg plastocyanin	2.9
Untreated membranes	22.1

Except for adding authentic spinach plastocyanin and using untreated membranes as indicated, experimental conditions were as described for the Zwittergent factor assay in fig.1

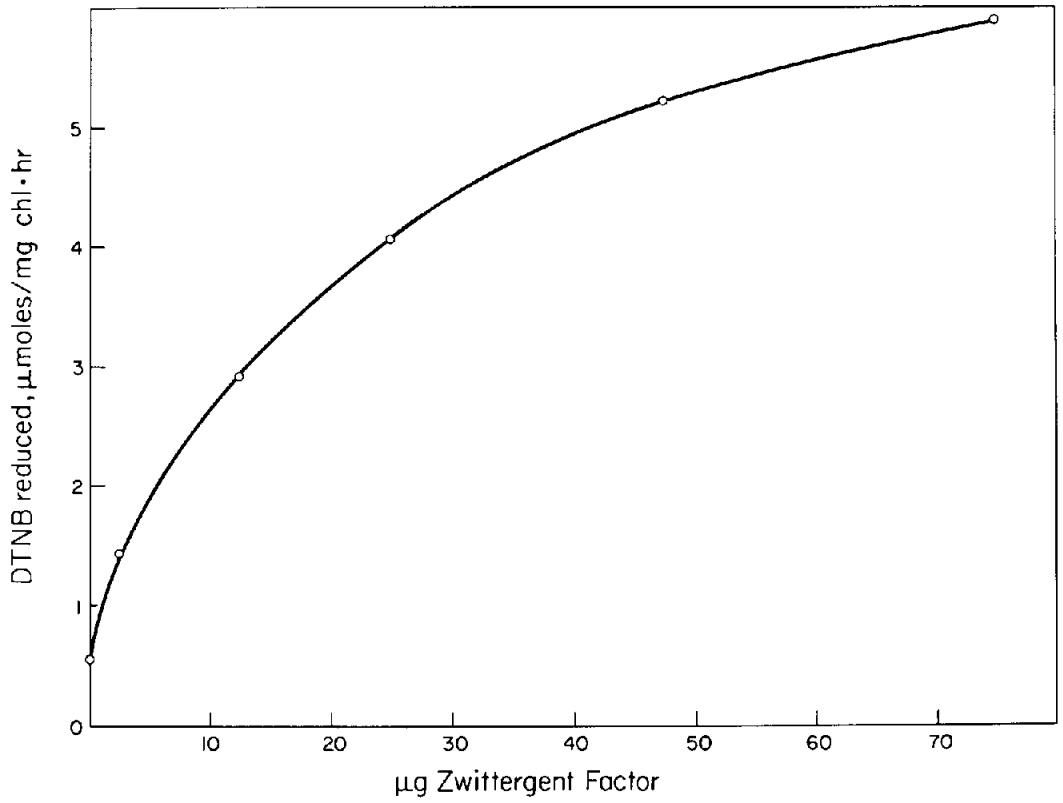

Fig.2. Effect of Zwittergent factor on photoreduction of DTNB by extracted spinach chloroplast membranes. Assays for photoreduction of DTNB were as in fig.1 for NADP except that ferredoxin, ferredoxin-NADP reductase, and NADP were omitted, DTNB (0.1 mM) was added, and absorbance changes were measured at 412 nm.

e.g., DTNB (fig.2) and methyl viologen (not shown). On the basis of these results, there appeared to be two possible sites for the Zwittergent factor to function in electron transport, viz., on the acceptor or the donor side of photosystem I. At the outset, we explored the first possibility and designed light/dark experiments to test this idea. In these experiments, we attempted to reduce substrate levels of the Zwittergent factor in the light and then to reduce an electron acceptor, such as ferredoxin or DTNB, in the dark. These experiments gave negative results irrespective of the electron acceptor tested.

We therefore turned our attention to the second possibility mentioned above (i.e., that the Zwittergent factor acted on the donor side of photosystem I). Our initial results obtained in this phase of the investigation were positive. We found that the photoreduction of an acceptor such as ferredoxin/NADP, which, as seen above, was dependent on the Zwittergent factor, required only ascorbate as electron donor without the need for DCPIP. This result suggested that the Zwittergent factor was either identical with or a substitute for plastocyanin, the membrane-bound blue copper protein of chloroplasts that is known to serve as a port of entry of ascorbate-based electrons in treated chloroplast membranes [16]. The identity of the Zwittergent factor with plastocyanin seemed a definite possibility in view of the fact that it, as is often the case with plastocyanin, was colorless when isolated. Plastocyanin is known to turn blue when oxidized by an oxidant such as ferricyanide.

3.3. *Identification of the Zwittergent factor as plastocyanin*

To test its relationship to plastocyanin, we added ferricyanide to the purified Zwittergent factor and then determined its optical and EPR spectra. As is the case with plastocyanin, ferricyanide added to the Zwittergent factor immediately elicited the appearance of a bright blue color that showed:

(i) An absorption spectrum with a peak in the visible region at 597 nm and in the ultraviolet at 280 nm;
(ii) An EPR spectrum with a g value at 2.05.

In related experiments, we observed that the purified Zwittergent factor contained a protein indistinguishable from authentic plastocyanin in native and SDS–polyacrylamide gel electrophoresis and in its reaction with plastocyanin rabbit antibody. [Both components showed a precipitin reaction in Ouchterlony double diffusion up to a 1:4 dilution of test antiserum (not shown)]. With this result, we concluded that the purified Zwittergent factor fraction contained plastocyanin as its protein component.

To confirm that solubilized plastocyanin was the biologically active agent, we carried out electron transport experiments in which authentic plastocyanin replaced the Zwittergent factor. These experiments revealed the interchangeability of the Zwittergent factor and plastocyanin in the photoreduction of NADP (table 1) and of the other electron acceptor used above, i.e., DTNB (not shown). These results thus show that plastocyanin is released from chloroplast membranes by Zwittergent extraction and is required as an intermediary between ascorbate and the photosynthetic electron-transport chain.

4. Concluding remarks

These results provide evidence that the Zwittergent-solubilized chloroplast component required for electron transport is plastocyanin. In accord with its known function, plastocyanin served as the port of entry of electrons (derived from ascorbate) to photosystem I with the treated membranes and was required for photosynthetic electron transport irrespective of the electron acceptor added (ferredoxin/NADP, methyl viologen, DTNB). These effects of the Zwittergent on photosynthetic electron transport reported above for spinach were, significantly, not peculiar to chloroplasts from this source. Similar results, with respect both to release of plastocyanin from the membranes and to its requirement for electron transport, were obtained with chloroplasts from peas. Thus, it seems that the Zwittergent reagent is generally effective in selectively releasing membrane-bound plastocyanin in chloroplasts from different sources, including peas, the source used in [5,6].

A final point to be made is that we obtained no evidence throughout this investigation for a protein disulfide component (e.g., a LEM) that functioned on the acceptor side of photosystem I (cf. [5,6]). Accordingly, if such a component exists and functions in chloroplasts, other procedures must be employed for its identification and solubilization.

Acknowledgements

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