ISOLATION AND PARTIAL CHARACTERIZATION OF MEMBRANE-BOUND FERREDOXIN-NADP$^+$-REDUCTASE FROM CHLOROPLASTS

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1. Introduction

Ferredoxin-NADP$^+$-reductase (EC 1.6.7.1), a FAD-containing protein, catalyzes the final step in the chain of reactions involved in photosynthetic NADP-reduction in chloroplasts [1–3]. After its discovery by Avron and Jagendorf [4] several laboratories have reported purification and characterization of the soluble enzyme from cell-free extracts or the stroma fraction of chloroplasts from higher plants and green algae [5–9]. Although the enzymic properties and molecular interactions with photosynthetic electron carrier proteins were intensively studied (see [10]), the molecular weight and subunit structure of the enzyme has been a matter of some confusion. Molecular weights of between 30–85 $\times$ 10^3 for the native conformation [4–6,8] and 37–45 $\times$ 10^3 for 1 subunit [5,11] or 2 dissimilar subunits [12] have been reported. Five isoenzymes of the stromal ferredoxin-NADP$^+$-reductase have beeh described [13]. To some extent reductase molecules are tightly bound to the thylakoid membranes. This has been concluded from the following facts:

(i) The ability of stroma-freed thylakoids to carry out in vitro NADP-reduction [3];
(ii) The production of reductase antibodies by immunization of rabbits with purified thylakoids [14];
(iii) Positive results of mixed antigen agglutination of thylakoids using antibodies against preparations of the stromal reductase [15,16].

Abbreviations: SDS, sodium dodecyl sulfate; FAD, flavin-adenine dinucleotide; DCIP, 2-6-dichloroindophenol

A ratio of 2 nmol membrane-bound reductase/1 nmol *P*-700 has been determined in stroma-free thylakoid membranes [17]. At present, however, it is not quite clear whether or not the stromal and membrane-bound ferredoxin-NADP$^+$-reductases are identical proteins. In order to get a better insight into the mechanism of photosynthetic NADP-reduction, detailed knowledge of the biochemical properties of these enzymes is essential.

Here we deal with the isolation and purification of the membrane-bound and stromal reductase and their characterization by means of structural and functional investigations. The membrane-bound enzyme is detached from thylakoids by EDTA or detergents. A procedure is described which yields a highly purified protein. In contrast to the stromal enzyme the solubilized reductase with NADP-specific diaphorase activities contains no FAD.

Immunological and gel electrophoretic experiments demonstrated that the apoproteins of the stromal as well as solubilized membrane-bound ferredoxin-NADP$^+$-reductase are structurally related and/or identical. Two-dimensional separation of both proteins on polyacrylamide gels indicates the presence of two distinct polypeptides with mol. wt 33 $\times$ 10^3 and 35 $\times$ 10^3, respectively. From the results it has been concluded that two distinct molecular weight classes of ferredoxin-NADP$^+$-reductase forms are linked to the thylakoid membranes of chloroplasts.

2. Materials and methods

Ferredoxin-NADP$^+$-reductase from *Spinacia oleracia* was purchased from Sigma (St Louis, MO).

A monospecific antiserum against the stromal ferredoxin-$NADP^+$-reductase from pinach was a gift from Dr R. J. Berzborn (Bochum, FRG). Ferredoxin-$NADP^+$-reductase from *Vicia faba* chloroplast stroma was prepared by a standard procedure [18], involving acetone fractionation, DEAE-cellulose chromatography and finally purification by chromatography on hydroxylapatite. Envelope- and strome-free chloroplast thylakoid membranes from *Vicia faba* were purified as in [19]. Determination of protein [20] and immunoelectrophoresis [21] were performed as described.

The NADP-specific diaphorase activity of ferredoxin-$NADP^+$-reductase in a spectroscopic test was assayed at 25°C with DCIP as electron acceptor. The reaction mixture contained the following components: 100 μl NADPH (mg/ml), 100 μl 0.1 M $MgCl_2$, 100 μl 1 M Tris–HCl (pH 7.6), 500 μl 0.2 M DCIP, 20 μl enzyme solution and 1 ml distilled water. The reaction was started by addition of NADPH and monitored at 600 nm with a Unicam SP 1800 recording spectrophotometer. One unit of activity is defined as the amount which catalyzes the reduction of 1 μmol DCIP/min at 25°C.

Gels were stained for diaphorase activity by incubation in 0.05 M Tris–HCl (pH 7.5) containing 0.4 mM DCIP. After 20 min incubation, 1 mg/ml NADPH was added to the solution. The presence of the enzyme was indicated by formation of a colourless band on a blue background [22].

Analytical slab or disc gels consisting of 9% acrylamide were run essentially as in [23]. The polypeptide composition of the proteins was analyzed by polyacrylamide gel electrophoresis in the presence of 0.1% SDS [24]. Gel slabs (7 × 10 × 0.3 cm) were prepared in cuvettes and consisted of 11% acrylamide containing 5 M urea. In two-dimensional gel electrophoresis the Davis gel was used in the first dimension and the 11% acrylamide slab gel, containing 5 M urea and 0.1% SDS, in the second dimension. The molecular weight analysis of reductase polypeptides using reference proteins were performed on split-gels as in [25].

3. Results and discussion

To release the tightly bound ferredoxin-$NADP^+$-reductase, purified thylakoid membranes were treated with various reagents, e.g., detergents and EDTA, which were known to be effective for membrane protein solubilization. The samples were centrifuged at 150 000 × *g* for 1 h to remove insoluble membrane material. Aliquot of the supernatants were separated by gel electrophoresis and checked for NADP-specific diaphorase activity. Detergents such as Na-deoxycholate, Triton X-100 or digitonin at >0.2% in 50 mM Tris–HCl buffer (pH 8.0) removed a substantial part of the diaphorase activity from the membranes. However, maximal solubilization was achieved by extraction with 1 mM EDTA (pH 7.0). Figure 1 shows that decreasing amounts of 1 component with diaphorase activity is solubilized by 3 successive EDTA washes of the same membrane pellet. A residual activity which remained still membrane-bound after EDTA treatment could be solubilized with 1% Triton X-100 or 1% Na-deoxycholate. Extractability of the enzyme by EDTA supports suggestions that ionic interactions are involved in the membrane attachment of the enzyme [12].

In order to purify the solubilized enzyme the EDTA extracts of thylakoid membranes were con-

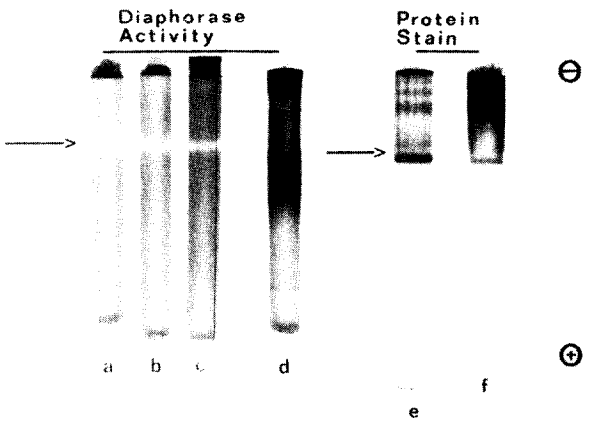

Fig.1. Extraction of membrane-bound ferredoxin-$NADP^+$-reductase. Stroma-freed chloroplast thylakoid membranes (1 mg chlorophyll) were extracted 3 times by incubation with 2 ml 1 mM EDTA (pH 7.0) (a–c) followed by 1 incubation with 2 ml 1% Triton X-100 in 50 mM Tris–HCl, pH 8.0 (d). The membrane material was precipitated by centrifugation after each step of extraction. Aliquots of the supernatants (100 μl) were separated electrophoretically on 9% polyacrylamide gels and checked for NADP-specific diaphorase activity (a–d) or stained for protein (e,f).

Table 1
Purification data of the EDTA-solubilized membrane-bound ferredoxin-NADP$^+$-reductase from *Vicia faba* thylakoid membranes

Fraction	Protein (mg)	Total act. units	Spec. act. units (mg^{-1})	Purif. factor	Recovery (%)
EDTA extract	153	6619	43.26	–	100
DEAE cellulose	37	3721	103.93	2.4	54
Bio-gel P 100	3.6	2026	563.02	13	30

centrated by ultrafiltration and applied to a DEAE (52)-cellulose column (2 × 40 cm) which had been equilibrated with 5 mM Tris–HCl buffer (pH 8.0). The fraction with diaphorase activity was eluted by a linear NaCl gradient at ~0.2 M NaCl, while the coupling factor of photophosphorylation (CF_1) remained on the column. After precipitation at 40–70% ammonium sulfate saturation the yellowish protein precipitate was dissolved in 5 mM Tris–HCl buffer (pH 8.0) and dialyzed against the same buffer. The dialyzed solution was applied to a Bio-gel P-100 column (2 × 70 cm) pre-equilibrated with the above-mentioned buffer. The results of a typical fractionation are shown in table 1.

The solubilized reductase as purified by the foregoing procedure was electrophoretically homogeneous. In gel electrophoresis following [23] the native protein moved essentially as the stromal ferredoxin-NADP$^+$-reductase isolated from *Spinacia oleracia* and *Vicia faba* (fig.2). Sometimes, two protein bands with very similar mobilities appeared during the gel electrophoresis. A homospecific antiserum against the stromal ferredoxin-NADP$^+$-reductase from spinach gave only one precipitation arc in the immunoelectrophoresis for the stromal as well as solubilized membrane-bound reductase from *Vicia faba* (fig.3). The electrophoretical mobility of both proteins was identical. Thus the products formed with the antibodies seem to be very similar and/or the same antigens which is a strong indication that both proteins are at least structurally related.

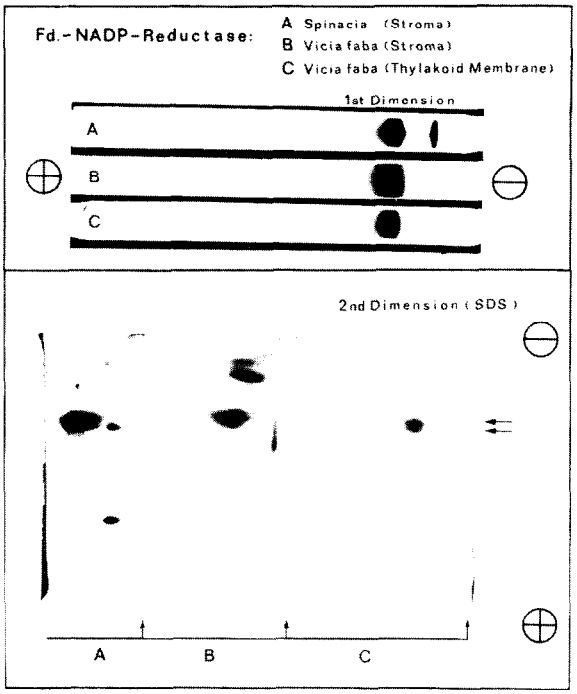

Fig.2. Two-dimensional polyacrylamide gel electrophoresis of partially purified stromal (A, B) and solubilized and purified membrane-bound (C) ferredoxin-NADP$^+$-reductases. Two-dimensional polyacrylamide/SDS–urea gel electrophoresis was performed as in section 2.

Fig.3. Immunoelectrophoresis of stromal (A) and solubilized membrane-bound ferredoxin-NADP$^+$-reductase (B) from *Vicia faba* chloroplasts. The antiserum used was against a stromal reductase preparation from spinach.

In order to check the purity and polypeptide composition of our reductase preparations the native proteins were separated electrophoretically using 9% cylindrical polyacrylamide gels followed by electrophoresis in the second dimension on 11% polyacrylamide slab-gels in the presence of 0.1% SDS with or without 5 M urea. As can be seen in fig.2, the stromal as well as the solubilized reductase were separated into two distinct polypeptides with slightly different electrophoretic mobilities. In the absence of urea both polypeptides showed essentially the same mobility during the gel electrophoresis. Molecular weights of reductase polypeptides had been determined by split-gel electrophoresis in the presence of 0.1% SDS and 5 M urea to be 33×10^3 and 35×10^3, respectively (fig.4). According to the binding of Coomassie brilliant blue R250 determined by densitometry of the stained gels the quantitative ratio of the polypeptides seems to be 1 : 1 in the case of the solubilized reductase in contrast to the stromal one. It was concluded [26] from these results that the reductases are composed of 2 non-identical subunits as in [12]. However, it is much more likely that the stromal as well as solubilized

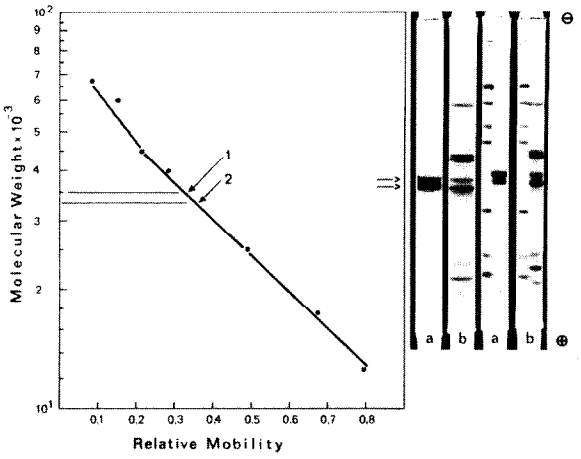

Fig.4. Molecular weight determination of polypeptides of the solubilized and purified membrane-bound (a) and stromal (b) ferredoxin-NADP$^+$-reductase from *Vicia faba* by SDS–polyacrylamide split-gel electrophoresis. Gel electrophoresis was performed as in section 2. The reductase preparations were incubated with 2% sodium dodecylsulfate and 1% 2-mercaptoethanol at 100°C for 3 min. Protein markers were carboxymethylated bovin serum albumin, ovalbumin, aldolase, catalase, chymotrypsinogen A, myoglobin and cytochrome *c*.

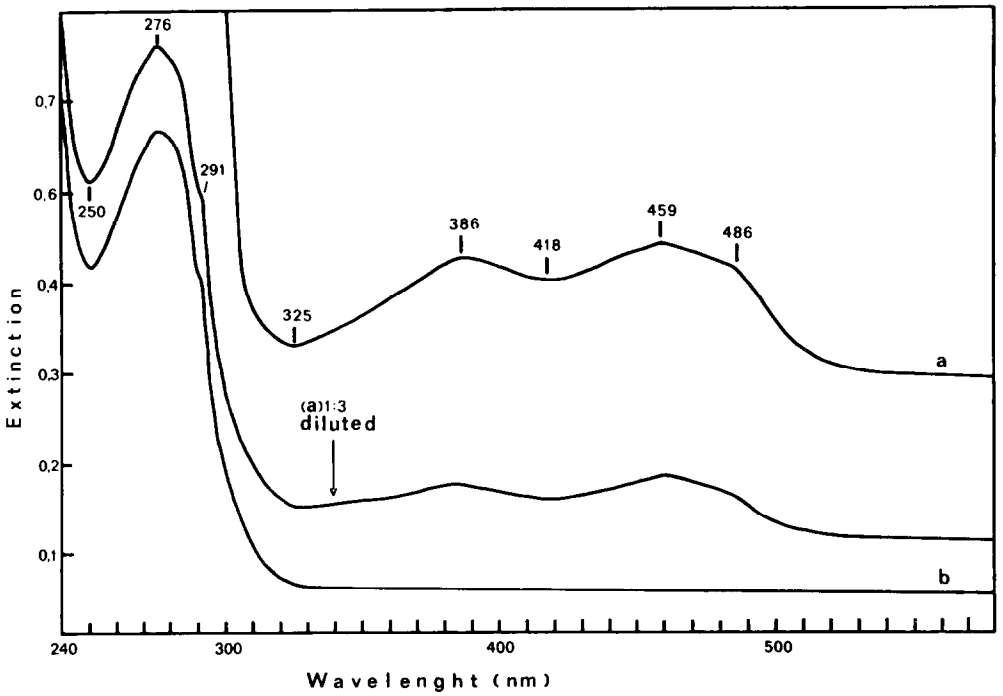

Fig.5. Absorption spectra for the stromal (a) and solubilized membrane-bound (b) ferredoxin-NADP$^+$-reductase from *Vicia faba* in 50 mM Tris–HCl (pH 8.0).

reductase preparations consist of 2 distinct molecular weight classes of ferredoxin-NADP$^+$-reductase forms as described for the stromal enzyme from spinach [13]. In good agreement with the present results these authors report mol. wt $33-34 \times 10^3$ or $36-38 \times 10^3$ for 4 of the 5 molecular forms of spinach reductase separated by isoelectric focusing.

Although the apoprotein of stromal and solubilized reductase are structurally related or identical (as shown by immunological experiments and the molecular weights of their polypeptides which are the same) the enzyme detached from the thylakoid membrane contained no detectable flavin by spectrophotometric analysis (fig.5). In contrast the partially purified stromal ferredoxin-NADP$^+$-reductases investigated showed absorption maxima at 386, 459 and 486 nm with a ratio of E_{276}/E_{459} of ~8. The reason why FAD is absent in the EDTA-solubilized and purified reductase is not clear at present. Dissociation of the non-covalently linked flavin from the apoprotein during extraction and/or purification may be possible. On the other hand, the membrane-bound reductase may not contain any prosthetic group and, therefore, needs a further membrane-bound cofactor for physiological activity. Loss in enzyme activity after solubilization supports the latter suggestion [12].

The ability of the EDTA-solubilized and purified reductase to catalyze the transfer of electrons from NADPH (but not NADH) to a variety of acceptors, including DCIP, ferricyanide and nitroblue tetrazolium, is of particular interest. However, it was shown [27] that the stromal ferredoxin-NADP$^+$-reductase from which FAD was removed by $CaCl_2$ treatment exhibited a rather low residual diaphorase activity. However, this might be due to a denaturation which made the protein enzymically inactive.

Summarizing, from the present study the apoproteins of stromal and membrane-bound ferredoxin-NADP$^+$-reductase can be considered to be identical or very similar. In contrast to the stromal reductases the solubilized and purified membrane-bound enzyme contains no FAD, but shows NADP-specific diaphorase activity.

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