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RESOLUTION AND RECONSTITUTION OF SPINACH FERREDOXIN-NADP$^+$ REDUCTASE

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SUMMARY. The apoprotein from spinach ferredoxin-NADP$^+$ reductase was prepared by treatment with 3 M calcium chloride. This procedure caused complete removal of the FAD prosthetic group together with considerable denaturation of the apoprotein. Thus, the recovery of total activity upon reconstitution with FAD was only 30%. More importantly, however, both transhydrogenase and diaphorase activities were 70% of that native enzyme based on bound flavin. The visible spectrum and properties of the reconstituted reductase were undiscernible from those of the native protein.

Ferredoxin-NADP$^+$ oxidoreductase (ferredoxin-NADP$^+$ reductase; EC 1.6.7.1, formerly EC 1.6.99.4) is an FAD-containing enzyme and functions as the terminal catalyst for NADP$^+$ reduction in photosynthetic electron transport (1,2,3). During the last decade the protein was mainly characterized by kinetic studies of its transhydrogenase and diaphorase activities including complex formation with ferredoxin (1-6; see [7] for review). Only recently have more direct approaches to its molecular structure been reported. Dansylation evidenced a lysyl group (8) and treatment with butanedione indicated an arginine residue as being responsible for binding of pyridine nucleotides at the active site (9).

It was shown previously that FAD is not covalently linked to the protein and data were presented indicating that the flavin is hidden within the protein structure ([10], see also [7]). Although many other flavoproteins could be resolved and reconstituted, all similar attempts to date with the reductase were unsuccessful. For example, apoproteins have been obtained

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from either FMN-containing flavoproteins like flavodoxins (11-13) or FAD-containing ones (14-16).

In this paper, we describe the preparation of the apoprotein of ferredoxin-$NADP^+$ reductase which could be reconstituted. Our preparative procedure follows the $CaCl_2$ method reported for deflavo-xanthine oxidase (16).

MATERIALS AND METHODS

Ferredoxin-$NADP^+$ reductase from spinach (Spinacia oleracea, strain Atlanta, grown in the open in thoroughly fertilized soil) was prepared according to the method of Forti (17). The enzyme was electrophoretically homogeneous with an apparent molecular weight of about 37,000 daltons as determined by sodium dodecylsulfate disc gel electrophoresis performed according to Weber and Osborn (18). The spectrum of the purified protein is shown in Figure 1 and characterizing data are given therein. The preparation of the apo-reductase from approximately 1.4 mg of holoenzyme is described in the RESULTS section.

Unless otherwise stated enzymatic measurements were carried out at room temperature (approx. 22 °C) in 0.08 M Tris-HCl, pH 7.5. The transhydrogenase assay contained 0.2 mM NADPH (as donor) and 0.025 mM thionicotinamide-$NADP^+$ (as hydrogen acceptor). In the diaphorase assay the nucleotide analog was replaced by 0.05 mM dichlorophenolindophenol.

Enzymatic activities are reported as the change of absorbance per 10 seconds associated with reduction of the electron acceptor used in the assay (3), and referred to an absorbance of 1.0 of the flavin peak at 458 nm (= A_{458}; see Table 1).

All spectra were recorded with a Varian Super-Scan 3 spectrophotometer; enzymic measurements were performed with a Hitachi spectrophotometer, model 181, and recorded with a W + W recorder 1100.

Pyridine nucleotides, the analog and FAD were purchased from Boehringer, Mannheim. Sephadex was obtained from Pharmacia. All other reagents were of analytical grade from Merck AG, Darmstadt, or Serva, Heidelberg.

RESULTS AND DISCUSSION

Morell (19) was the first to report the dissociation of protein-bound FAD from xanthine oxidase after treatment with $CaCl_2$. Following this procedure Komai et al. (16) successfully reconstituted xanthine oxidase from the apoenzyme and FAD. They reported that dissociation of protein-bound flavin depends on the concentration of $CaCl_2$, temperature and time. In our experiments, the best conditions for preparing reconstitut-

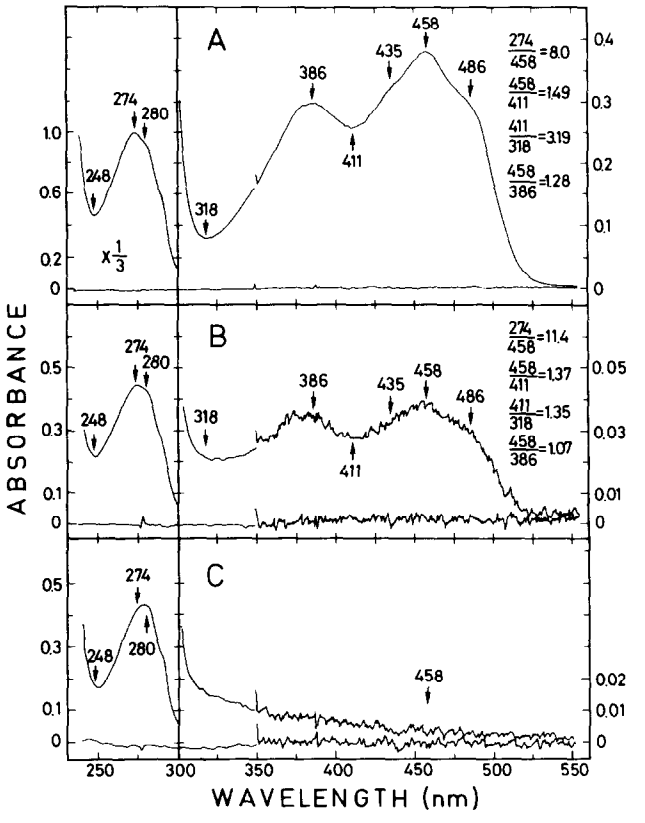

Figure 1 - Absorption spectra of the original ferredoxin-NADP$^+$ reductase (A; diluted 3-fold in the UV region), reconstituted holoenzyme (B), and apo-reductase (C). Spectra were recorded in the elution buffer which served also as reference. Right ordinate applies to spectra in the visible region. Curves reported are for a typical experiment; each experiment was repeated three times.

able apoenzyme of the spinach reductase were incubation with 3 M $CaCl_2$ for 2 hours at 10 °C in the presence of 1 mM dithiothreitol (DTT) and 0.1 mM EDTA. DTT was added to avoid oxidation of thiol groups, which seem to be responsible for a pH-dependent transition between low and high activity forms of the enzyme (20).

Complete chromatographic separation of free flavin from the apoprotein was achieved with Sephadex G-25; the column (1 x 15 cm) was prior equilibrated with 0.01 M Tris-HCl buffer, pH 7.5, including 1 mM DTT and 0.1 mM EDTA (Fig.2). Aliquots from each fraction were assayed for transhydrogenase activity

Table 1 - Enzymatic activities of native and reconstituted ferredoxin-NADP$^+$ reductase.

Enzyme preparation	(1) Transhydrogenase $\frac{A_{400}}{10 \text{ sec} \times A_{458}}$	(2) Diaphorase $\frac{A_{600}}{10 \text{ sec} \times A_{458}}$	(3) Ratio of (2):(1)
Native enzyme	543 (100%)	409 (100%)	0.75
Reconstituted enzyme	373 (69%)	291 (71%)	0.78

ΔA = absorbance change at 400 or 600 nm, respectively (see text). Percentage of specific activity is given in brackets.

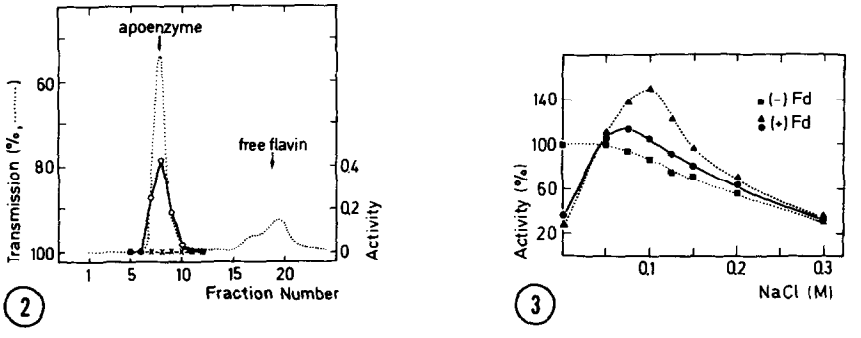

Figure 2 - Separation of the ferredoxin-NADP$^+$ reductase apoprotein from its FAD prosthetic group by Sephadex G-25 chromatography. The elution was recorded with an LKB UviCord II, measuring transmission at 280 nm. The activites of the eluted protein fractions were determined by the transhydrogenase assay without (X—X) or in the presence (O—O) of 0.05 mM FAD and expressed as absorbance change at 400 nm per min and aliquot (10 µl) of each fraction.

Figure 3 - Effect of NaCl and ferredoxin (Fd) on diaphorase activity of the reconstituted reductase. Besides dichlorophenolindophenol each reaction mixture contained: Tris-HCl, pH 7.3, ionic strength = 0.01; NADPH 0.025 mM; spinach ferredoxin 0.3 µM (●—●) or 1.5 µM (▲···▲); reconstituted ferredoxin-NADP$^+$ reductase 12 nM; NaCl was added as indicated, ferredoxin was a pure preparation with an absorbance ratio of A_{420}/A_{275} = 0.48.

to determine the degree of resolution. It should be noted, however, that after $CaCl_2$ treatment and subsequent Sephadex chromatography a rather low residual activity was sometimes observed but which is negligible with respect to the degree of reactivation of the apoenzyme. Addition of FAD to the apo-reductase fractions restored the enzymatic activity (Fig.2). Those fractions exhibiting the best reconstitution were pooled and concentrated by adding course dry Sephadex G-25.

For isolation of the reconstituted reductase, the apo-reductase was incubated with a 10-fold excess of FAD (0.5 mM at 4 °C) for 8 hours in the dark. The reconstituted holoenzyme was separated from excess free flavin by passage through a Sephadex G-25 column (equilibrated with 10 mM Tris-HCl, pH 7.5). The spectrum of the apoenzyme given in Figure 1 clearly demonstrates that the FAD prosthetic group of the ferredoxin-$NADP^+$ reductase is completely lost by the $CaCl_2$ treatment. The maximum absorbance of the holoenzyme is at 274 nm but is shifted to 280 nm in the apoenzyme. Further, the shoulder near 283 nm, present in the native enzyme, disappears as well as the typical flavin absorption in the visible region.

Compared to the native enzyme, the reconstituted holoenzyme had a specific activity of 70% for both the transhydrogenase and diaphorase reactions based on the flavin absorbance at 458 nm (Table 1). Further, the ratios of the two activities for both proteins are essentially equal (col. 3).

As shown in Figure 3, the diaphorase activity is depressed approximately 75% in the presence of ferredoxin at low ionic strength (no added NaCl). This inhibition indicates complex formation between the reductase and ferredoxin as was earlier proposed by Nakamura and Kimura (4). Further, the effect of higher ionic strength was similar for both the native and the reconstituted reductase (data only shown for the latter). At about 0.1 M NaCl the well-known stimulation of activity is observed (3).

A comparison of the spectral data of the native enzyme with the reconstituted enzyme shows that the ratio of the absorbance at 274 nm to 458 nm, which is often used as a criterion of purity, is considerably increased (from 8 to 11.4). This finding indicates that not all of the apoenzyme could be subsequently reassociated with FAD. At present, this method

allows for only a 30% recovery of total enzymatic activity. (The recovery reported herein was about 20% since only the main fractions of the Sephadex elution steps were collected.) The comparatively low recovery is an apparent disadvantage of the method. However, since the flavin prosthetic group is thought to be buried in the protein molecule (8), one would expect a high degree of denaturation as apparently the protein has to unfold to allow for release of the FAD.

The lowered specific activity of the reconstituted enzyme (70%) can be explained by the binding of FAD to a modified form of the apoprotein which remains inactive even after recombination. Alternatively, it could be due to a lowered turnover number for the reconstituted reductase. Further data are necessary to distinguish between these two possibilities.

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