SOLUBILIZATION FROM SPINACH THYLAKOIDS OF A HIGHER MOLECULAR WEIGHT FORM OF FERREDOXIN-NADP$^+$ REDUCTASE

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1. Introduction

Until recently, it was generally accepted that the flavoprotein ferredoxin-NADP$^+$ reductase (EC 1.6.7.1), a membrane-bound component of the photosynthetic electron-transport chain, as purified from spinach leaves, was a monomer of mol. wt 4×10^4 [1]. However, several laboratories presented conflicting data on the existence of polymeric forms of this enzyme. Higher molecular weight forms of the reductase, detectable in spinach leaves crude extracts but not in purified preparations of FNR, were first reported in [2]. However, a rather stable heterodimer of the flavoprotein was claimed isolated by the usual purification procedure in [3]. In agreement with [2], the absence of polymeric forms was demonstrated in pure preparations [1]. However, in [4] and for algal FNR [5] ~10% of a homodimer of the reductase was found in highly purified preparations. In [4,5] substantially modified purification procedures were used.

Thus, it is not clear whether ferredoxin-NADP$^+$ reductase exists in polymeric forms in the native state or whether these aggregates arise during purification; also, the nature of the higher molecular weight enzymes is still not understood. Here the existence of polymeric forms of ferredoxin-NADP$^+$ reductase in spinach leaf crude preparations is ascertained by using highly specific tests (i.e., ferredoxin reductase activity and immunological methods) and by using a variety of extraction conditions. The loss of these forms during the usual purification procedure is also demonstrated. Further, we report on the different solubilization characteristics of the dimer and on some of its properties.

Abbreviations: FNR, ferredoxin-NADP$^+$ oxidoreductase; INT, 2(*p*-iodophenyl)-3-nitrophenyl-5-phenyltetrazolium chloride; DCPIP, 2,6 dichlorophenolindophenol; SDS, sodium dodecylsulfate; β-SH, β-mercaptoethanol; PMSF, phenylmethylsulphonyl fluoride

2. Materials and methods

Activity measurements were performed at 25°C in 1 ml final vol. Reductase activity with ferredoxin or $K_3Fe(CN)_6$ was tested essentially as in [6]. Diaphorase activity with INT or DCPIP was followed at 490 nm and 600 nm, respectively, by replacing in the ferricyanide assay medium the electron acceptor with 0.2 mM INT or 50 μM DCPIP. Spinach crude extracts were obtained by homogenizing in a Waring blender 100 g leaves with 2 vol. extraction solution (see table 1). After filtration through 8 layers of cheese cloth, the homogenate was fractionated between 40–75% $(NH_4)_2SO_4$ immediately, unless otherwise stated. The precipitate was redissolved in 50 mM Tris–HCl/50 mM NaCl (pH 7.5) (4°C), dialyzed and applied on a calibrated Sephadex G-100 column (2.2 × 95 cm) equilibrated with the same buffer. The eluted fractions were tested for activity and the amount of the enzyme quantitatively determined by immunodiffusion according to [7]. The antibody used was a purified γ-globulin fraction, obtained by ammonium sulfate fractionation of a rabbit antiserum raised against pure ferredoxin-NADP$^+$ reductase. SDS electrophoresis was carried out in a polyacrylamide gradient pore gel slab as in [8].

3. Results and discussion

3.1. *Gel filtration analyses of crude preparations of FNR*

The presence in crude extracts of spinach leaves of higher molecular weight forms of ferredoxin-$NADP^+$ reductase has been ascertained by following a more specific assay of the reductase, i.e., the NADPH-ferredoxin reductase activity with cytochrome *c* as final acceptor and by specific immune precipitation with the antibody raised against the pure flavoprotein. In fig.1, 3 peaks of ferredoxin-$NADP^+$ reductase are shown of $M_r > 100\,000$, 70 000 and 34 000. Fractions from the 3 peaks gave complete identity with pure FNR as determined by Ouchterlony double diffusion method. It can be seen that measuring diaphorase activity with ferricyanide or DCPIP as electron acceptors as some authors did, tends to overestimate the peak of M_r 70 000 (or to pick out a non-existent peak of FNR) due to the presence in spinach leaves or even chloroplast extracts of high quantities of an enzyme which has diaphorase activity but does not interact with ferredoxin. This contaminant has been specifically identified as glutathione reductase. By using INT as electron acceptor a more reliable test for the diaphorase activity of FNR can be devised, still not completely specific.

Following the observation [4] that, by leaving the spinach homogenate in 40% $(NH_4)_2SO_4$ for many hours (≤50), the diaphorase activity of the solution

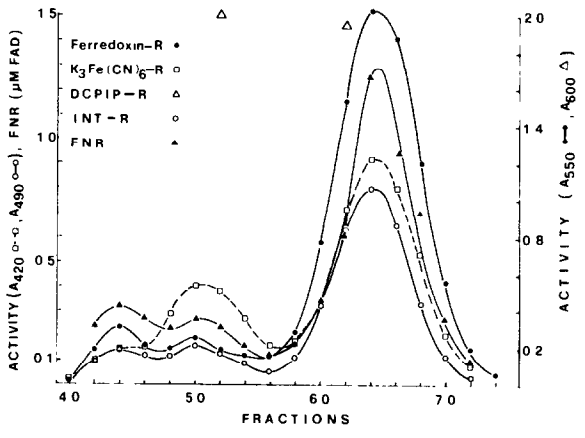

Fig.1. Elution pattern from Sephadex G-100 of a FNR preparation made as in table 1 (c_I). The activity is expressed as $\Delta A \cdot min^{-1} \cdot 50\ \mu l^{-1}$. The FNR was quantitated by immunodiffusion as in section 2.

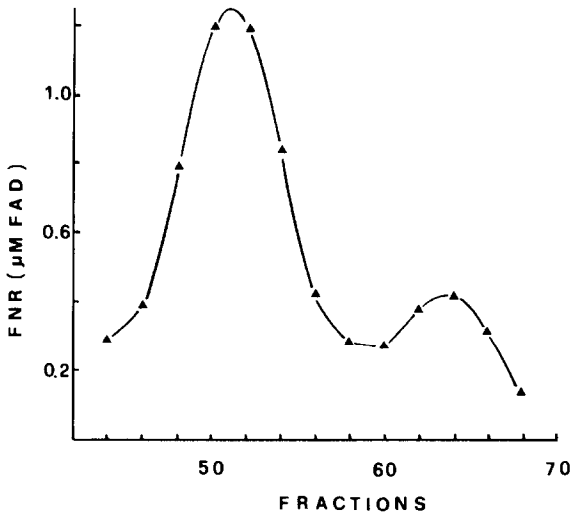

Fig.2. Elution pattern from Sephadex G-100 of a FNR preparation made as in table 1 (e_{II}). Other conditions were as in fig.1.

increased considerably, we treated the precipitate in the same way at 40% ammonium sulfate, left after a preliminary extraction of the FNR. The pattern obtained by gel filtration of the enzyme thus extracted is depicted in fig.2. A remarkable increase of the form of M_r 70 000 was observed. A closer examination of the data of fig.1 reveals that activities referred to mol enzyme-flavin as determined by immunodiffusion, are considerably lower (30–50%) in the peaks of high molecular weight in comparison to those in the monomer peak. This was consistently found in all the experiments reported in table 1. Quantitative data on the recovery of the two major forms of the FNR by different types of extraction are summarized in table 1. It shows that a treatment of spinach leaves with buffer at low ionic strength (table I, c_I) solubilized only about 30–40% of the FNR, mostly in monomeric form. By using more drastic extractions (table 1, e_I,b,a) the amount of FNR released was noticeably increased, the ratio between the two major peaks being ~1. By treating as described in table 1 (c_{II} or e_{II}) the thylakoid membranes pre-washed to release the flavoprotein (table 1, c_I or e_I), more ferredoxin-$NADP^+$ reductase could be solubilized with a net increase in the amount of the form of M_r 70 000 (ratio 67–77 to 33–23 of monomer). When the preparative procedure of [1] was used, we extracted 9600 units enzyme [1], a further extraction of the

Table 1
Solubilization of FNR from spinach leaves by different extraction conditions and its distribution in the two major M_r classes

Extraction conditions		FNR[a] total	FNR[a] (%)	
			M_r 70 000	M_r 34 000
(a)	2% Triton, 0.1 M Tris–HCl (pH 8) (5 h at 4°C)	90	45	55
(b)	2 mM Tris–30 mM NaCl, 40% $(NH_4)_2SO_4$ (50 h at 4°C) [4]	80	50	50
(c_I)	2 mM Tris–30 mM NaCl [4]	35	30	70
(c_{II})	40% $(NH_4)_2SO_4$ precipitate after (c_I) extraction, treated as in (b)	58	67	33
(d)	(c_I) + (c_{II})	93	53	47
(e_I)	2 mM Tris, 30 mM NaCl, 30 mM $MgCl_2$, 10 mM β-SH, 1 mM EDTA, 10 μM PMSF, 40% $(NH_4)_2SO_4$, (3 h at 4°C)	63	51	49
(e_{II})	40% $(NH_4)_2SO_4$ precipitate after (e_I) extraction, resuspended in (e_I) medium, (50 h at 4°C)	37	77	23
(f)	(e_I) + (e_{II})	100	60	40

[a] Determined by immunodiffusion as in section 2

40% ammonium sulfate precipitate as in table 1 (c_{II}) released another 2400 units. It should be noted that the presence in the extraction medium of 2% Triton X-100 or 10 mM β-mercaptoethanol (maintained also during gel filtration) did not eliminate the high molecular weight forms (table 1). However, rechromatography of the 70 000 M_r species concentrated by ultrafiltration, on the same G-100 column yielded a conspicuous amount of monomer. This should be one of the reasons why the 70 000 M_r form is found only in small amounts [4,5] or not at all [1] in highly purified enzymes. Additional reasons are the following:

(1) More drastic conditions of extraction should be used to recover the 70 000 M_r form in high yield (table 1) than those usually employed in most purification procedures;

(2) The acetone treatment as used by the more common purification procedures of the FNR [1,9,10] eliminates this form from the main fraction.

3.2. *Some properties of the FNR solubilized from the thylakoid membrane*

Ferredoxin-NADP$^+$ reductase has been solubilized, on a preparative scale, from the 40% $(NH_4)_2SO_4$ sediment left after a standard preparation of the enzyme [1] and a procedure has been devised (to be published elsewhere) which yielded two fractions of FNR both ~85–90% of purity. One fraction (FNR I) contained the enzyme as a monomer, the other one (FNR II) had a high content of the 70 000 M_r species. From the spectra depicted in fig.3, which are similar to those in [11], one can infer that the FNR solubilized from spinach thylakoids is certainly a flavoprotein. This is at variance with the report [12] about an FNR solubilized from *Vicia faba* thylakoids which has diaphorase activity but no flavin bound. The SDS-electrophoresis in a gradient pore gel slab revealed a single major band for both FNR I and II, and these bands showed the same migration.

3.3. *Conclusions*

From these data we can conclude the following:

(i) Existence in crude extracts of spinach leaves of a

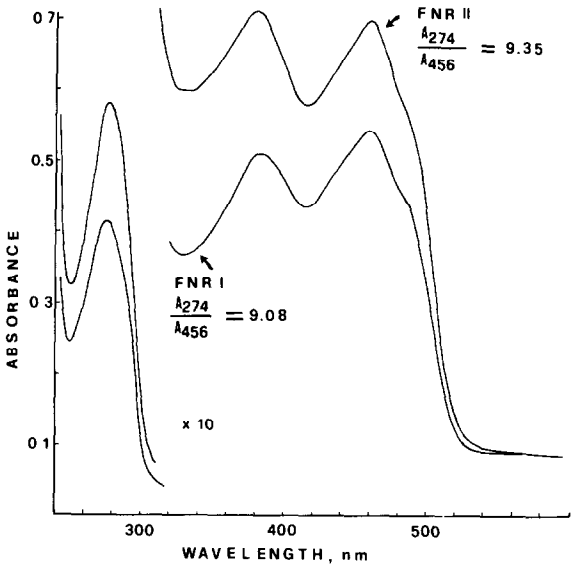

Fig.3. Visible and ultraviolet spectra of FNR, partially purified from the thylakoid membranes.

70 000 M_r species of ferredoxin-NADP$^+$ reductase, which is a homodimer as deduced from SDS electrophoresis and spectral data;

(ii) The dimer tends to dissociate and it is usually lost during the standard purification procedure;

(iii) All the FNR forms solubilized from washed thylakoids are flavoproteins, in contrast to the findings in [12];

(iv) The differential way of solubilization of the dimer implies either a diverse binding to the membrane or a different localization;

(v) The dimer is less active than the monomer under the same assay conditions.

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