PURIFICATION OF FERREDOXIN-NADP⁺ REDUCTASE, FLAVODOXIN
AND FERREDOXIN FROM A SINGLE BATCH OF THE
CYANOBACTERIUM *Anabaena* PCC 7119

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Methods are described for the simultaneous isolation of ferredoxin-NADP⁺ reductase, ferredoxin and flavodoxin from large quantities of the cyanobacterium *Anabaena* PCC 7119 allowing the use of a single batch of cells. The ultraviolet-visible spectra and the extinction coefficients of ferredoxin-NADP⁺ reductase and ferredoxin were determined. The purification procedure also yields enriched fractions of phycobiliproteins and cytochrome c553.

INTRODUCTION

Ferredoxin-NADP⁺ reductase (FNR) (E.C. 1.18.1.2) is an FAD-containing enzyme which catalyzes the reduction of NADP⁺ using reducing equivalents generated by the photosynthetic apparatus. It was first isolated from spinach¹ and subsequently from different cyanobacteria including *Anabaena*²⁻⁴. Ferredoxin is a small iron-sulfur electron-carrier protein which performs the transfer of electrons between photosystem I and FNR and is involved in many other biological electron transport chains in cyanobacteria⁵. It has been purified from plants, algae and cyanobacteria^{2,6}. Flavodoxin is a low molecular weight FMN-containing flavoprotein which, while constitutive in some species, has been found to be synthesized in some organisms to replace ferredoxin as a consequence of the iron-

limiting conditions in the culture medium⁷. It has been isolated from different cyanobacteria^{8,9}.

Phycobiliproteins have been purified from cyanobacteria¹⁰. They are highly specialized antenna pigments and their function in these organisms is similar to that of chlorophylls. Cytochrome c553 is functionally analogous to plastocyanins and donates electrons to photosystem I¹¹.

All the proteins mentioned above (except phycobiliproteins) participate, in cyanobacteria, in reactions that involve transfer of electrons. This is accomplished after formation of stable complexes between the redox partners. It is very important, then, to study those structural features which affect the electron transfer reaction. Since large amounts of proteins are required for those studies (X-ray crystallography and NMR spectroscopy), a procedure for fast, easy isolation of sufficient amounts of the proteins is required.

In the present paper we describe the combined purification of FNR, ferredoxin and flavodoxin from *Anabaena* PCC 7119 as well as the production of highly enriched fractions of phycobiliproteins and cytochrome c553 from the same organism. The procedure described here represents an improvement over existing procedures for the purification of such proteins since it allows the use of a single batch of cells as the starting material.

MATERIALS AND METHODS

Cell Growth. *Anabaena* PCC 7119 cells were grown autotrophically on nitrate as described¹², using an intermediate iron concentration (2,6 μM); iron was provided to the cultures as ferric ammonium citrate. The culture was bubbled with a mixture of 5% (v/v) CO_2 and air and lit with a bank of fluorescent light tubes that gave an intensity of 4000 lux at the surface of the cultures. Cells were harvested by centrifuging at the end of the exponential phase of growth and stored at -20°C .

Purification Procedure. The general scheme of purification is shown in Figure 1. All steps were carried out at 4°C using 50 mM Tris-HCl buffer, pH 8.0

FIGURE 1.

General procedure for purification of proteins from *Anabaena*.

containing 0.1 mM EDTA, 0.1 mM mercaptoethanol and 1 μ M PMSF. From the second DEAE-cellulose chromatography step onwards, 50mM Tris-HCl, pH 8.0 was used.

Cells (2 kg wet weight) were thawed and suspended in buffer (1: 2 w/v), and acetone (precooled to -20°C) was added with vigorous stirring to give a final concentration of 80%. After 10 min the mixture was filtered through a porous plate and the precipitate washed with 9 liters of precooled acetone and finally with 1 liter of diethyl ether. The blue residue was allowed to dry at room temperature overnight. The homogenate was prepared by suspending 350 g of the acetone powder in buffer (1:10 w/v), stirring for 120 min and centrifuging for 30 min at 35,000 x g. The supernatant, which is referred to as the crude extract, was brought to 35% saturation with solid $(\text{NH}_4)_2\text{SO}_4$. After centrifugation the precipitate was discarded and the supernatant was brought to 65% $(\text{NH}_4)_2\text{SO}_4$ saturation and centrifuged again. The precipitate, which contains the FNR, was resuspended in 1 liter of buffer, dialyzed against the same buffer, mixed with 1 liter of "settled" DEAE-cellulose (Whatman DE-52) and gently stirred for 90 min. The suspension was poured into a 15-cm diameter column and washed with 1 liter of buffer. FNR and most of phycobiliproteins were eluted with buffer containing 0.15 M NaCl. The pooled fractions of FNR activity were dialyzed and applied to a Cibacron Blue-Sepharose¹³ column (4,5 x 26 cm). Phycobiliproteins were not retained and were obtained in the washing volume. FNR was eluted with a linear gradient (0-0.5 M) of NaCl, dialyzed against 50 mM Tris-HCl pH 8.0 and applied to a DEAE-cellulose column (2,5 x 50 cm). After washing, FNR was eluted with a linear gradient (0-0.15M) of NaCl and the pooled fractions were dialyzed and applied to a second Cibacron Blue-Sepharose column (3 x 25 cm), washed and eluted with a linear gradient (0-0.5 M) of NaCl to yield homogeneous enzyme.

The brownish supernatant from the 65% $(\text{NH}_4)_2\text{SO}_4$ precipitation, which contained flavodoxin, ferredoxin and cytochrome c, was applied to a DEAE-cellulose column (4.5 x 40 cm), previously equilibrated with 65% $(\text{NH}_4)_2\text{SO}_4$ in buffer, and was washed and finally eluted with a reverse gradient (65-0 %) of $(\text{NH}_4)_2\text{SO}_4$. Well separated fractions containing cytochrome c, flavodoxin and ferredoxin were eluted. The flavodoxin-containing fraction was dialyzed against 50 mM Tris-HCl pH 8.0 and applied to a DEAE-cellulose column, washed and

eluted with a linear gradient (0-0.5M) of NaCl. After dialysis, the pooled flavodoxin fractions were applied to a 2 cm diameter column composed by Sephacryl S-200 (65 cm) and an upper layer (5 cm) of DEAE-cellulose. After washing, flavodoxin was eluted with 50 mM Tris-HCl buffer pH 8.0 containing 0.5 M NaCl. Homogeneous protein was obtained by repeating the direct gradient DEAE-cellulose chromatography step with a smaller column (1 x 15 cm).

The pooled ferredoxin-containing fractions were dialyzed against 0.2 M acetate buffer, pH 5.5, applied to a DEAE-cellulose column (2 x 40 cm) previously equilibrated in the same buffer, washed and eluted with a linear gradient (0.2-1 M) of acetate buffer pH 5.5. Eluted ferredoxin was concentrated in an Amicon ultrafiltration cell to a 5 ml volume and was incubated with 1 mg/ml DNAase and RNAase at 15 °C for 24 hours. After this treatment, it was applied to a Sephadex G-50 column (2 x 55 cm) and eluted with 50 mM Tris-HCl buffer pH 8.0. The pooled ferredoxin fractions were applied to a DEAE-cellulose column (1 x 15 cm), washed and eluted with a linear gradient (0-0.5 M) of NaCl, to yield homogeneous protein.

General Methods. Quantitative protein determinations were performed by the Lowry procedure¹⁴. Purified protein concentrations were determined from the visible absorption spectra using the following extinction coefficients: Flavodoxin $\epsilon_{464} = 9.4 \text{ mM}^{-1} \text{ cm}^{-1}$ (9) FNR $\epsilon_{458} = 9.4 \text{ mM}^{-1} \text{ cm}^{-1}$ and ferredoxin $\epsilon_{423} = 7.2 \text{ M}^{-1} \text{ cm}^{-1}$. The extinction coefficient of FNR was determined from the absorption changes detected on releasing the bound FAD from the enzyme by heating. The extinction coefficient of ferredoxin was calculated by determination of the iron content of a sample (whose spectrum had been prerecorded), using a Perkin-Elmer Plasma 40 emission spectrometer, assuming a 2Fe-2S prosthetic group. Spectra were recorded using a Kontron Model 860 spectrophotometer. Antibodies against ferredoxin and flavodoxin were prepared¹⁵ and immunoelectrophoresis performed using the method described by Laurell¹⁶ to quantify the amount of both proteins in the purification steps. FNR was quantified during purification by determining the diaphorase activity using dichlorophenolindophenol¹⁷.

TABLE I

Purification Table of *Anabaena* PCC 7119 FNR.

Purification step	Volume (ml)	Protein (mg)	Activity (I.U.)	Yield (%)	Purification (fold)
Crude extract	4500	95850	46350	100	1
65 % Precipitate	800	62320	38950	84	1.3
Batch DEAE	1740	12180	37125	80	6.3
Cibacron Blue I	825	701	30770	66	91
DEAE-cellulose	255	316	24149	52	158
CibacronBlueII	86	189	22201	48	243

RESULTS AND DISCUSSION

The purification procedure reported here provides pure homogeneous FNR, flavodoxin and ferredoxin from *Anabaena* PCC 7119 as well as highly purified fractions of phycobiliproteins and cytochrome c, thus avoiding the use of different batches of the cyanobacterium to purify each protein.

The data in Table I show a yield close to 50% and a 250-fold purification for FNR, quite similar to the values reported by Sancho *et al.*⁴ for *Anabaena variabilis* PCC 6309, the purification procedure being essentially the same. The main advantage of this method is the removal of most of the phycobiliproteins in one step by means of the Cibacron-Blue Sepharose chromatography. Another alternative method has been described³ using affinity chromatography on 2',5'-ADP-Sepharose. It also provides good results but has the disadvantage of its higher cost. Shin and Oshino¹⁸ described a purification of FNR using ferredoxin-Sepharose 4B but the yields obtained with that method are considerably lower. The purified enzyme preparation showed only a single band on SDS-polyacrylamide gel electrophoresis (see Fig 2, lane C) when stained with Coomassie blue and exhibited a spectral absorption ratio (A_{274}/A_{458}) of 7.90, whereas Serrano and Rivas reported³ a value of 8.26. Figure 3 shows the absorption spectrum of FNR. It is very similar to that of *Anabaena variabilis* or

FIGURE 2

SDS-polyacrylamide gel electrophoresis of the purified proteins from *Anabaena* PCC 7119. Lane A: molecular weight standard proteins: phosphorylase b (94,000), bovine serum albumin (67,000), ovoalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and α -lactalbumin (14,000). Lane B: 30 μ M flavodoxin. Lane C: 30 μ M FNR. Lane D: 12 μ M ferredoxin

spinach FNR and shows absorption maxima at 274, 392 and 458 nm and minima at 317 and 417 nm. An extinction coefficient of 9.4 $\text{mM}^{-1} \text{cm}^{-1}$ at 458 nm was calculated for *Anabaena* PCC 7119 FNR, similar to the *Anabaena variabilis* enzyme coefficient ($\epsilon_{459} = 9.7 \text{ mM}^{-1} \text{cm}^{-1}$ (4)).

The phycobiliprotein-enriched fraction eluted from the first Cibacron Blue-Sepharose column can be further purified¹⁰ using chromatography on hydroxyapatite to yield phycoerythrin, phycocyanin and allophycocyanin.

Purification of *Anabaena* PCC 7119 flavodoxin was based on the method described by Fillat *et al.*⁹. Table II shows that the protein was purified 175-fold with a 63% yield. This method includes, as the initial step, an ammonium sulfate fractionation which provides an essentially phycobiliprotein-free supernatant containing flavodoxin, ferredoxin and cytochrome c. We have found 65% $(\text{NH}_4)_2\text{SO}_4$ to be the optimal concentration, slightly lower than that used by Fillat *et al.*⁴. At higher values some of the flavodoxin precipitates, while at lower values

FIGURE 3.

UV and visible spectrum of ferredoxin-NADP⁺ reductase from *Anabaena* PCC 7119 in 50 mM Tris/HCl buffer, pH 8.0.

TABLE II

Purification Table of *Anabaena* PCC 7119 flavodoxin.

Purification step	Volume (ml)	Protein (mg)	Flavodoxin (mg)	Yield (%)	Purification (fold)
Crude extract	4500	95850	562	100	1
Supernatant	4660	6480	466	83	12
DEAE reverse grad	360	1330	433	77	57
DEAE direct grad. I	155	530	399	71	132
Sephacryl S-200	60	372	365	65	172
DEAE direct grad. II	24	354	354	63	175

FIGURE 4.

Chromatography on DEAE-cellulose in 65% ammonium sulfate. Elution profile of proteins from a column treated with a reverse gradient 65%-0% ammonium sulfate in 50 mM Tris/HCl buffer, pH 8.0. Peak 1: cytochrome c; peak 2: flavodoxin; peak 3: ferredoxin.

part of the FNR stays in solution. This procedure, designed to avoid phycobiliprotein contamination, has been used previously by Hutber *et al.*¹⁹ in the purification of flavodoxin and ferredoxin from *Nostoc*. The next purification step used by these authors is a DEAE-cellulose chromatography eluted with a direct salt gradient. The use of a reverse $(\text{NH}_4)_2\text{SO}_4$ gradient avoids dialysis against a different buffer and gives good separation of three different fractions containing cytochrome c, flavodoxin and ferredoxin respectively (Figure 4).

Gel filtration of the fraction containing flavodoxin posed a problem due to the large amount of protein present (approx. 400 mg), as a consequence of the relatively high mass of cyanobacteria used. This drawback was avoided by using a small layer of DEAE-cellulose on top of a Sephacryl S-200 column. Eluting with a saline buffer, concentration and gel filtration were performed in a single step thanks to the strongly anionic character of flavodoxin.

Pure flavodoxin showed a single band on SDS-polyacrylamide gel electrophoresis (see Fig 2, lane B) and exhibited the same absorption ratio

TABLE III

Purification Table of *Anabaena* PCC 7119 ferredoxin.

Purification step	Volume (ml)	Protein (mg)	Ferredoxin (mg)	Yield (%)	Purification (fold)
Crude extract	4500	95850	194	100	1
Supernatant	4660	6480	176	91	13
DEAE reverse grad	495	820	155	80	93
DEAE / acetate	112	248	142	73	283
Nucleases+Seph G-50	45	139	111	57	394
DEAE / NaCl	22	105	105	54	494

($A_{274}/A_{465} = 5.88$) and spectral properties as have been previously described for this flavodoxin⁹.

The data in Table III show an approximate 54% yield with a 500-fold purification for *Anabaena* PCC 7119 ferredoxin. Only one reddish-brown band, corresponding to this protein was observed in all purification steps and purified ferredoxin showed a single band in SDS-polyacrylamide gel electrophoresis (see Fig 2, lane D) which migrates in an anomalous fashion in this medium as has been described elsewhere²⁰. This indicates the presence of a single type of ferredoxin in *Anabaena* PCC 7119 when this cyanobacterium is grown at an intermediate iron concentration (2.6 μ M). Two different ferredoxins, I and II, are present in *Anabaena variabilis* PCC 6309²¹ and in *Nostoc* strain MAC²² and *Nostoc muscorum*²³, which are genetically close to *Anabaena* PCC 7119, when they are grown in iron-rich media. Using the above-mentioned iron concentration we obtained a flavodoxin/ferredoxin ratio of 2.9, which is similar to that described for *Anabaena* ATCC 29413²⁴. While flavodoxin has been reported to replace ferredoxin I in *Anabaena*⁹ this does not occur in the conditions described here where some iron is still present in the culture medium. It has been reported for *Nostoc* strain MAC²² that ferredoxin II decreases more rapidly with iron deprivation, and is virtually absent in iron-free media.

FIGURE 5.

UV and visible spectrum of ferredoxin from *Anabaena* PCC 7119 in 50 mM Tris/HCl buffer, pH 8.0.

Purifications of ferredoxins from *Anabaena variabilis*^{2,6} and *Nostoc*²² have been described. An initial ammonium sulfate fractionation eliminates most phycobiliproteins. This step is usually followed by a DEAE-cellulose chromatography. We used a reverse gradient of $(\text{NH}_4)_2\text{SO}_4$ to elute the proteins. The suitability of this method to separate flavodoxin, ferredoxin and other proteins was first described by Mayhew and Howell²⁵. Some ferredoxins from cyanobacteria have been reported to be contaminated with nucleic acids. *Anabaena* PCC 7119 ferredoxin presented considerable contamination of this kind, which made it necessary to use DNAase and RNAase after a DEAE-cellulose chromatography in acetate buffer to provide the optimum pH for nuclease activity.

Figure 5 shows the absorption spectrum of *Anabaena* PCC 7119 ferredoxin. It has maxima at 275, 330, 423 and 463 nm and minima at 293, 391 and 453 nm; the absorption spectral ratio (A_{275}/A_{423}) was 1.75. This ratio is slightly better than the 1.79 obtained for *Nostoc muscorum*.²³ The absorption coefficient at 423 nm was calculated to be $7.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

A cytochrome-containing fraction is eluted from the DEAE-cellulose column with a reverse $(\text{NH}_4)_2\text{SO}_4$ gradient. This cytochrome is isolated in its reduced form and its absorption spectrum shows an α -band at 553 nm which identifies it as a photosynthetic cytochrome. This protein has been reported to replace plastocyanin in some cyanobacteria in copper-deficient media¹², while it is always present in other species, constitutively incapable of synthesizing plastocyanin¹¹. As *Anabaena* PCC 7119 was grown in $1 \mu\text{M}$ copper, it seems to belong to the second group. This cytochrome can be further purified by chromatography in carboxymethyl-cellulose².

The procedure described here permits the isolation of different photosynthetic proteins from the same batch. Some of these proteins have already been cloned in *E. coli*^{26, 27, 28} and their expression in this bacteria is an excellent way to obtain them. However the growth of cyanobacteria provides the possibility of using ^{13}C -labelled CO_2 or ^{15}N -labelled ammonium chloride, the proteins thus obtained being most useful for NMR and other studies requiring labelled proteins.

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