Isolation of Phytoflavin, A Flavoprotein with Chloroplast Ferredoxin Activity

Robert M. Smillie

Biology Department, Brookhaven National Laboratory, Upton, New York

During recent years considerable attention has been focused on the role of non-haem proteins containing iron in biological electron transfer systems. Several proteins of this type showing identical catalytic properties have been isolated from green cells (6). These proteins, which are localized in the chloroplasts, include the methaemoglobin reducing factor (5) from parsley, photosynthetic pyridine nucleotide reductase (17) from spinach, and the rote Ferment (7) from Chlorella. All catalyze the photoreduction of NADP by chloroplasts.

A protein possessing similar properties has been found in anaerobic bacteria. This protein has been named ferredoxin (14). Like the chloroplast protein, ferredoxin contains iron and sulfur as the only known constituents other than protein. Ferredoxin functions as an electron carrier in nitrogen fixation (13), the synthesis (1) and cleavage (15) of pyruvate, formate oxidation (3), and in several hydrogenase coupled reactions including the reduction of hydroxylamine (24), NADP (22) and the oxidation of hypoxanthine (23).

Ferredoxin also supports the photoreduction of NADP by chloroplasts (21). Conversely, the related chloroplast protein can replace bacterial ferredoxin in the hydrogenase system of Clostridium pasteurianum (21). For these reasons Tagawa and Arnon (21) have proposed that the term ferredoxin should also include the non-haem iron containing protein found in chloroplasts. This terminology will be adopted here, although it is to be noted that chloroplast and bacterial ferredoxins are not identical and differ in their absorption spectra, molecular weight and the number of labile iron and sulfur atoms per mole protein.

The isolation of a new protein showing chloroplast ferredoxin activity is reported in this paper. This protein is not a ferredoxin. Since it contains FMN and mediates in the photoreduction of NADP by chloroplasts isolated from a variety of higher plants and algae, it has been named phytoflavin. The biological properties of phytoflavin will be described in a subsequent paper.

A preliminary report of this work has appeared (20).

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Materials and Methods

**Growth of Cells.** Cells of Anacystis nidulans were grown in 10-liter bottles each containing 3 liters of the medium D prepared by Kratz and Myers (12). The bottles were positioned horizontally on a shaker platform inside a New Brunswick incubator. The cultures were illuminated by an overhead bank of white fluorescent lights. The light intensity at the surface of the cell cultures was between 6000 and 10,000 lux. The temperature of the chamber was maintained at 37°C. The cultures were gassed with 5% CO₂ in air and were shaken continuously. Each culture was started with a 5% inoculum and was allowed to grow to maximum cell density (3-5 days) before being harvested.

**Assay for Chloroplast Ferredoxin Activity.** Chloroplast ferredoxin activity was assayed by a spectrophotometric method as described previously (19). The reaction mixture contained washed chloroplasts (18 μg chlorophyll), Tris-HCl pH 7.8 (20 mM), MgCl₂ (2.5 mM) and NADP (110 μM). The final volume was 0.8 ml and the assay temperature was 25°C. Chloroplasts were prepared from the leaves of 9- to 12-day-old pea plants (Pisum sativum L.) following the procedure used for spinach by San Pietro and Lang (17).

**Results**

**Separation of Proteins with Chloroplast Ferredoxin Activity.** Cells of A. nidulans were harvested by centrifugation and washed twice with 0.05 M Tris pH 7.8. The washed cells were stored in a deep freeze until a sufficient quantity was accumulated. In a typical experiment, 100 to 200 g wet weight of cells were suspended in 0.05 M Tris pH 7.8 and were disrupted by passage through a French pressure cell at 20,000 p.s.i. The mixture was centrifuged at 144,000 × g for 60 minutes. Solid ammonium sulfate was added to the dark blue supernatant fluid to a final concentration of 60%. After standing at 0°C for 15 minutes the suspension was centrifuged at 10,000 × g for 15 minutes. The supernatant fluid was retained and ammonium sulfate was added to saturation. The resulting precipitate was collected by centrifugation at 10,000 × g for 15 minutes. This precipitate was dissolved in 0.05 M Tris pH 7.8 and was dialysed against 0.005 M Tris pH 7.8. Most of the chloroplast ferredoxin activity was precipitated between 60 and 100% saturated ammonium sulfate (fraction A, Table 1). A further fraction containing chloroplast ferredoxin activity was recovered by

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3 Present address: Plant Physiology Unit, C.S.I.R.O. and School of Biological Sciences, University of Sydney, Australia.
Allowing the saturated ammonium sulfate solution to stand at 2°C for 1 week. The brown precipitate that formed was collected by centrifugation and dialysed against 0.005 M Tris pH 7.8 (fraction B).

**Chromatography of Fraction A.** Fraction A was purified further by chromatography on a column of DEAE-cellulose. After dialysis against 0.05 M K-phosphate buffer pH 7.5, fraction A was absorbed onto a column which had been equilibrated against the same buffer. The column was washed with 0.1 M phosphate buffer pH 7.5. A yellow fraction was then eluted with 0.25 M phosphate buffer. A second fraction, brown in color, was eluted with 1 M phosphate buffer. The second colored fraction was identified as ferredoxin by its absorption spectrum. However, the activity of this fraction accounted for only about 50% of the original activity of fraction A. Accordingly, the yellow fraction was tested for chloroplast ferredoxin activity. It was found to be very active and in fact accounted for most of the remaining chloroplast ferredoxin activity (table I). Depending upon the batch of cells used, the ratio of the activities of the yellow fraction and the ferredoxin fraction varied from 1:1 to 1:5.

In subsequent experiments the 2 fractions with chloroplast ferredoxin activity were separated by gradient elution from a DEAE-cellulose column. Figure 1 shows the separation of colored proteins in a 65 to 100% saturated ammonium sulfate fraction from a cell-free extract of *A. nidulans*. A higher starting concentration of ammonium sulfate (65%) was used in this experiment to ensure complete removal of the phycocyanins. This resulted in some loss of the first fraction containing chloroplast fer-

### Table I. Fractionation of Proteins with Chloroplast Ferredoxin Activity

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Chloroplast ferredoxin activity (μmol NADP* reduced per min)</th>
<th>Activity per mg protein</th>
<th>Purification*</th>
</tr>
</thead>
<tbody>
<tr>
<td>60-100% sat. (NH₄)₂SO₄ (fraction A)</td>
<td>578</td>
<td>0.714</td>
<td>17</td>
</tr>
<tr>
<td>Precipitate from 100% sat. (NH₄)₂SO₄ supernatant fluid after standing 7 days. (fraction B)</td>
<td>120</td>
<td>4.73</td>
<td>115</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography of fraction A: 0.1 M-0.25 M phosphate</td>
<td>252</td>
<td>4.31</td>
<td>105</td>
</tr>
<tr>
<td>0.25 M-1.0 M phosphate</td>
<td>272</td>
<td>5.29</td>
<td>128</td>
</tr>
</tbody>
</table>

* These values are based on an activity of 0.041 per mg protein for the crude extract. They are approximate since it is difficult to obtain an accurate measurement of the activity of the crude extract.

Redoxin activity. The protein (350 mg) was absorbed onto a 15 cm × 0.9 cm column of DEAE-cellulose previously equilibrated with 0.005 M Tris-HCl pH 7.8. Protein was eluted by an increasing non-linear gradient of NaCl. A mixing chamber contained 400 ml of 0.005 M Tris pH 7.8. The reservoir contained 0.6 M NaCl in 0.005 M Tris pH 7.8. Upon commencing the elution a yellow fraction was rapidly removed from the column. The absorption spectrum of this fraction indicated that the color was due to pteridine. Two brown-colored fractions were then eluted. These were identified spectroscopically as cytochromes. The first cytochrome to be eluted had a λₚ at 555 mμ and appeared to correspond with the cytochrome that is rapidly oxidised upon illuminating *Anacystis* cells (16). The second cytochrome was identified as the low potential C-type cytochrome recently isolated by Holton and Myers (8). None of these fractions showed chloroplast ferredoxin activity. As the salt concentration was increased, 2 well-separated fractions showing chloroplast ferredoxin activity were obtained. The first was yellow in color, the maximum visible absorption occurring at 465 mμ. This fraction was identified with the yellow fraction described above that was eluted with 0.25 M phosphate. The second was brown in color, the maximum visible absorption oc-

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Fig. 1. DEAE-cellulose column chromatography of a 65 to 100% saturated ammonium sulfate fraction from an extract of *A. nidulans*. Solid lines show the absorbancy of different fractions at the wavelengths indicated. Dashed lines and the triangles indicate chloroplast ferredoxin activity in μmol NADP reduced per minute per ml fraction. The dotted line indicates the salt gradient (conductivity measurements). The absorbancy and activity values shown for the ferredoxin fractions are one-fifth the actual values obtained.
Occurring at 423 μm. It can be seen from figure 1 that the activity of each fraction coincided with the colored component of the fraction.

Absorption Spectra of Fractions with Chloroplast Ferredoxin Activity. Figure 2 shows the absorption spectrum of the first of the 2 active protein fractions obtained by the gradient elution with NaCl (fig 1). The absorption spectrum of the first fraction (phytoflavin) did not resemble the absorption spectrum of either chloroplast or bacterial ferredoxin. A trough occurred in a region of the spectrum where ferredoxins show visible absorption maxima (420 μm for chloroplast ferredoxin and 390 μm for bacterial ferredoxin). The absorption spectrum is typical of a flavoprotein. Maxima occurred at 377, 465 and 492 μm. The ultraviolet absorption showed a peak at 279 μm. The ratio of the absorption at 279 μm to 465 μm was 5.9.

The absorption spectrum of the second fraction showing chloroplast ferredoxin activity is shown in figure 3. It is characteristic of the absorption spectrum of the ferredoxin found in chloroplasts. This chloroplast-type ferredoxin is probably identical with a protein showing chloroplast ferredoxin activity that has been isolated from *A. nidulans* by Black et al. (2).

Absorption Spectrum and Chromatography of Fraction B. The activity of fraction B (see table 1) in the chloroplast assay system was due to the chloroplast-type ferredoxin. The absorption spectrum was very similar to that shown in figure 3. Only a single peak of chloroplast ferredoxin activity was obtained after chromatography of fraction B on DEAE-cellulose using gradient elution with NaCl.

Distribution of Fe⁵⁵ in Protein Fractions from *A. nidulans*. From figure 1 it is evident that there was a good correlation between the chloroplast ferredoxin activity and the flavoprotein content (as indicated by the absorbancy at 465 μm) of the phytoflavin fractions. However, the possibility remained that the activity of these fractions was not due to the flavoprotein but rather to the presence of a second peak of either chloroplast- or bacterial-type ferredoxin whose color was masked by that of the flavoprotein. Since the activity of ferredoxin in electron transfer systems is dependent upon its iron content, an experiment was carried out to determine if the phytoflavin fractions contained amounts of iron comparable to those present in the ferredoxin fractions. Cells were grown in the usual way except that Fe⁵⁵ was included in the growth medium. The cells were harvested in the late logarithmic phase of growth and were washed 3 times with 0.05 M Tris pH 7.8. The cells were disrupted in the French pressure cell and the extract obtained was fractionated between 60 and 100% saturated ammonium sulfate. The precipitated protein was dissolved, dialyzed and chromatographed on a DEAE-cellulose column using a NaCl gradient as described above. Figure 4 shows the separation of phytoflavin and ferredoxin from the column as well as the distribution of Fe⁵⁵. A peak of gamma-emitting activity coincided with the distribution of ferredoxin. Tubes containing phytoflavin had only small amounts of radioactivity. It should be noted that amounts of phytoflavin and ferredoxin having equal absorbancies at their respective visible absorption maxima (465 and 423 μm) showed the same order of activity in the chloroplast assay (fig 1). The possibility that the activity of the phytoflavin fraction was due to a combination of phytoflavin and a small amount of ferredoxin is also deemed unlikely. Both proteins appeared to act independently in mediating the photoreduction of NADP by chloroplasts and mixtures of the 2 gave additive results.

Stability of Phytoflavin. Phytoflavin is fairly stable at neutral pH. It is routinely stored frozen at
Fig. 4. Distribution of Fe^{55} in phytoflavin and ferredoxin fractions. The fractions were eluted from a DEAE-cellulose column by an increasing gradient of NaCl. The absorbancy values at 465 m{\mu} show the distribution of phytoflavin and those at 423 m{\mu} show the distribution of ferredoxin.

−20°. Only a small decrease in activity occurred after storage at this temperature for 1 year.

Identity of the Flavin in Phytoflavin. The yellow color of phytoflavin is due to bound FMN. Upon heating phytoflavin the visible spectrum changed to that of a flavin cofactor, i.e. the 465 m{\mu} maximum shifted to 450 m{\mu} and the 492 m{\mu} shoulder disappeared. The flavin group was split from the protein by heat, acidification, or digestion with a mixture of trypsin and chymotrypsin. The flavin group was identified as FMN by paper chromatography using 3 different solvent systems and by bioassay with apocytochrome c reductase (10). Flavin adenine dinucleotide was not detected.

Removal of FMN by acidification to pH 4 resulted in the loss of activity of phytoflavin. However, it has not been demonstrated conclusively that FMN is necessary for the activity of phytoflavin.

Acid Labile Sulfur. Spinach ferredoxin contains acid labile sulfur (6). It was shown that ferredoxin from A. nidulans also contains acid labile sulfur, but the latter was not detected in a 13 \mu{\text{m}} solution of phytoflavin. Acid labile sulfur down to 0.3 \mu{\text{m}} should have been detected by the assay system employed.

Discussion

Blue-green algae do not contain chloroplasts but possess lamellar structures which show some similarity to the photosynthetic apparatus of certain photosynthetic bacteria. In common with green plants they evolve O_{2} during photosynthesis. The experiments of Black et al. (2) and those described in this paper show that the major ferredoxin present in the blue-green alga A. nidulans is the chloroplast-type ferredoxin and not the type found in anaerobic bacteria including the photosynthetic bacterium Chromatium (9).

Fry and San Pietro (6) have shown that ferredoxins isolated from different plant sources exhibit minor differences in their absorption spectra. The absorption spectrum of the ferredoxin from A. nidulans corresponds more closely with the spectrum of the methaemoglobin reducing factor from parsley than with the spectrum of photosynthetic pyridine nucleotide reductase from spinach. The absorption spectra of the ferredoxin from A. nidulans and the methaemoglobin reducing factor lack shoulders at 290 m{\mu} and have minima at 295 m{\mu}, whereas the spinach protein shows a shoulder at 290 m{\mu} and a minimum at 305 m{\mu}. These differences in the ultraviolet absorption spectra are attributed to tryptophane, which is present in photosynthetic pyridine nucleotide reductase, but is absent in methaemoglobin reducing factor (6).

A second protein showing chloroplast ferredoxin activity has been purified from A. nidulans. A similar protein has been isolated from Anabacna cylindrica (unpublished experiments). This protein, phytoflavin, differs from other proteins with chloroplast ferredoxin activity that have been isolated previously from either plants or bacteria. The phytoflavin present in extracts of A. nidulans is eluted from a DEAE-cellulose column prior to the elution of a chloroplast-type ferredoxin. Phytoflavin contains FMN, whereas this cofactor is not present in either chloroplast or bacterial ferredoxin. The absorption spectrum of phytoflavin and the experiment with Fe^{55} indicate that the activity of phytoflavin cannot be attributed to contaminating ferredoxin. Since the extinction coefficients of chloroplast ferredoxin [\Sigma_{430} = 9.9 (6)] and FMN [\Sigma_{440} = 12.2 (4)] are similar, it can be seen from figure 1 that the activities of the 2 proteins from A. nidulans are comparable on a molar basis. The exact physiological role of phytoflavin remains to be determined.

Phytoflavin is not to be confused with ferredoxin-NADP reductase (18) [also known as pyridine nucleotide transhydrogenase (11)], the only other flavoprotein known to function in a photosynthetic electron transfer system. The reductase is the terminal enzyme in the electron transfer pathway of chloroplasts resulting in the photoreduction of NADP (18). This enzyme has an absorption spectrum (11) similar to phytoflavin, but contains flavin adenine dinucleotide (7, 11). Ferredoxin-NADP reductase shows pyridine nucleotide transhydrogenase activity as well as NADPH-diaphorase activity. It does not support the reduction of NADP by isolated chloroplasts in the absence of chloroplast ferredoxin. A
similar enzyme has been found in extracts of A. nidulans (unpublished experiments), but it is removed during the purification of phytoflavin. As will be shown in a subsequent paper, phytoflavin has neither pyridine nucleotide transhydrogenase nor pyridine nucleotide diaphorase activities.

Summary

Two proteins showing chloroplast ferredoxin activity have been separated by DEAE-cellulose chromatography of an extract of the blue-green alga Anacystis nidulans.

One of the proteins was identified as a chloroplast-type ferredoxin.

The other protein, named phytoflavin, could not be identified with either a chloroplast- or a bacterial-type ferredoxin. It catalyzes the photoreduction of nicotinamide adenine dinucleotide phosphate by washed chloroplasts in the absence of added ferredoxin. The flavin component of phytoflavin is flavin adenine mononucleotide.

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Literature Cited