Isolation and characterization of two different flavodoxins from the eukaryote *Chlorella fusca*

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Two different molecular forms of flavodoxin from the green alga *Chlorella fusca* have been purified to homogeneity and their properties compared. The molecular masses are 22 kDa (flavodoxin I) and 20 kDa (flavodoxin II). Western blots of axenic crude extract show the two bands. Both are single polypeptide chains and their N-terminal sequences differ but are very similar. Each form contains 1 mol of FMN/mol of apoprotein, exhibits a typical flavodoxin u.v.–visible absorption spectrum and does not contain covalently bound phosphate. The oxidation–reduction properties of the FMN in the flavodoxins differ considerably. Redox potentials of flavodoxin I at pH 8 are −240 mV for the oxidized/semiquinone couple and −350 mV for the semiquinone/hydroquinone couple. Flavodoxin II gives more electronegative values: −278 mV and −458 mV respectively. Flavodoxin II fulfils better the redox requirements for photosynthetic electron transport and, as expected, it is more efficient at mediating $NADP^+$ photoreduction in the photosynthetic electron flow. A new h.p.l.c. method for flavodoxin purification is described, which is useful for the isolation of very similar anionic proteins.

INTRODUCTION

Iron is an essential element of cytochromes and iron–sulphur proteins, which are components of the electron-transfer chains involved in important processes such as respiration, photosynthesis and nitrogen fixation. In spite of its natural abundance in aqueous environments, the poor solubility of the iron oxides in aqueous media at neutral pH could drastically limit the growth of phytoplankton [1]. It is well documented that conditions of iron limitation induce many physiological changes without significantly affecting the viability of cells. This adaptative response allows the organism to survive in environments with limited amounts of iron available. Under conditions of iron stress, the levels of all the iron-containing redox proteins associated with photosystems I and II and the cytochrome b_6–f complex are decreased [2]. One of the most impressive adaptive responses to iron starvation is the replacement of ferredoxin by the flavoprotein flavodoxin. It is generally accepted that flavodoxins replace ferredoxins in a wide range of reactions in which the latter participate [3].

Flavodoxins are small FMN-containing redox proteins which function at low redox potential in biological electron-transfer reactions. They have been considered to be prokaryotic proteins, with only a few exceptions: *Chlorella fusca* [4] and two red algae, *Chondrus crispus* [5] and *Porphyra umbilicalis* [6]. The crystal structure of *Chondrus* flavodoxin has recently been obtained and the differences in residues in the FMN-binding site correlated with their anomalous oxidized/semiquinone redox potential [7]. In some organisms (e.g. *Azotobacter vinelandii*) flavodoxin is a constitutive protein but, in the majority of cells, flavodoxin synthesis occurs only during growth in iron-deficient conditions. In *Chondrus*, flavodoxin has been described as constitutive and its metabolic role is still unknown [5], whereas in *Chlorella* it is induced in response to iron starvation and thought to replace ferredoxin in the electron-transfer chain associated with photosystem I [4].

It can be assumed that the role of flavodoxin in eukaryotic cells is to act as a substitute for ferredoxin allowing these cells to adapt to natural aquatic environments which are iron-deficient. The simultaneous presence of different ferredoxins in the same organism has been documented not only for bacteria [8], but also for cyanobacteria [9] and higher plants [10]. The presence of different molecular species of flavodoxin in one organism has been documented in *A. vinelandii* (strain 475) [11] and *Azotobacter chroococcum* [12], where two genetically distinct flavodoxins have been isolated with different physiological roles. Recently in *P. umbilicalis* [6], two forms have been described with different molecular masses but the same N-terminal sequence. In *Desulfovibrio gigas*, a new flavodoxin-like protein has been described [13], which allows *in vitro* the reconstruction of an electron-transfer chain from molecular hydrogen to sulphite reduction. This protein, called flavoredoxin, occurs simultaneously with flavodoxin.

We report here the characterization of the two flavodoxin isoforms found in large-scale preparations of *Chl. fusca* grown under conditions of iron deficiency. This study investigates the physiological role of these two forms of flavodoxin, their significance in eukaryotic cells and the relationship with flavodoxins from other sources.

MATERIALS AND METHODS

Organism

The green alga *Chl. fusca* Shihira et Krauss 211–215 was obtained from the University of Göttingen (Germany) culture collection.

Culture media and conditions

Cells were grown in batch cultures at 28 °C as described by Kessler and Czygan [14] in 10-litre bottles bubbling with a 5% CO_2 in air mixture illuminated by a bank of fluorescent light bulbs. In order to purify the flavodoxin, iron-deficient cells were cultured using 0.18 μM iron. Chlorophyll content was determined spectrophotometrically as described by Parsons and Strickland [15].

Abbreviation used: FNR, ferredoxin–$NADP^+$ reductase.
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Protein purification

In a typical purification, 200 g of cells grown in iron-limited conditions were disrupted with 1600 g of glass beads (200–300 μm) in 150 ml of 50 mM Tris/HCl, pH 8, containing 1 μM phenylmethanesulphonyl fluoride. Unbroken cells and debris were removed by centrifugation at 18000 *g* for 20 min (4 °C). The resulting supernatant was precipitated with 90% precooled acetone (−20 °C), and the pellet obtained after centrifugation at 18000 *g* for 10 min was dried. Acetone powders were resuspended in the homogenization buffer and stirred for 90 min in a cold-room. After centrifugation for 10 min at 18000 *g*, the resulting supernatant was applied to a DEAE-cellulose column (4 cm × 30 cm) equilibrated with 50 mM Tris/acetate, pH 8. Flavodoxin was eluted in a gradient of 0–0.5 M NaCl. The yellow fractions with a ratio of A_{280}/A_{460} less than 20 were pooled, and after a 1:3 dilution, were applied to a DEAE-cellulose column (3 cm × 40 cm) equilibrated with the same buffer. Finally, the flavodoxins were eluted with 50 mM Tris/acetate, pH 8, containing 0.22 M NaCl. The flavodoxin peak shows two bands on SDS/PAGE and the pooled fractions were repurified by h.p.l.c. on a Hydropore-5-SAX column (Rainnin). The flavodoxins were isolated using hydrophobic interaction conditions in a gradient from 3 M $(NH_4)_2SO_4$ in 0.02 M potassium phosphate, pH 7 (buffer A) to 100% of 0.02 M potassium phosphate, pH 7 (buffer B) over 35 min. Fractions were collected and ratios of A_{280}/A_{460} less than 5.4 for the first peak and 6 for the second were considered to be pure. Purity was confirmed by SDS/PAGE, and an absorption coefficient of 10000 $M^{-1} \cdot cm^{-1}$ at 462 nm was used to calculate the concentration of the samples [4].

Ferredoxin–$NADP^+$ reductase (FNR) from *Chl. fusca* was purified as previously described [16] from fractions collected after the first DEAE-cellulose column.

Immunological assays

Antibodies against the two proteins were prepared using pure proteins in New Zealand White rabbits [17]. Immunoquantification of flavodoxin was performed by rocket immunoelectrophoresis using the method described by Bog-Hansen [18].

Western-blot analysis

Western-blot analysis was performed using antibodies against flavodoxins I and II simultaneously. Horseradish peroxidase was used as the detection system. The samples were run on SDS/20% polyacrylamide gels (PhastSystem; Pharmacia) and transferred to nitrocellulose filters. The membranes were immunodecorated with the two antibodies followed by anti-rabbit IgG–peroxidase conjugate (Boehringer Mannheim). Dioctyl sulphosuccinate/3,3′,5,5′-tetramethylbenzidine substrates (Sigma) were used as the detection system.

Analytical procedures

Phosphorus content was determined by the method of Bartlett [19]. N-terminal sequence analysis for flavodoxin I was carried out at the microsequencing facility at Emory University (Atlanta, GA, U.S.A.) and for flavodoxin II at the Protein and Nucleic Acid Chemistry Center of Woods Hole Oceanographic Institution (MA, U.S.A.). Protein determinations were performed by the Lowry procedure [20].

Oxidation–reduction potential measurements

These were performed as described [21] at 25 °C in 50 mM Tris/acetate, pH 8. Reductant ($Na_2S_2O_4$) or oxidant {$K_3[Fe(CN)_6]$}, maintained under anaerobic conditions, was added with a microsyringe. The degree of reduction of the flavodoxin was monitored spectrophotometrically by following absorbance changes at 462 (or 464 nm) and 590 nm. Anthraquinone-2-sulphonate (5 μM; E_m −225 mV) and 5 μM Methyl Viologen (E_m −449 mV) were used as mediators. Flavodoxin reduction was monitored in a thermostatically controlled spectrophotometer equipped with a cell-stirring attachment.

Prosthetic group determination and quantification

These were performed by h.p.l.c., using a Spherisorb 5 μm and 80Å-C18 column (250 mm × 4.6 mm) from Kontron Instruments. Ammonium acetate, pH 6, at 0.1 M (solvent A) and methanol (solvent B) were used as eluents in a linear gradient from 0 to 60% of solvent B in 25 min.

Photoreduction of $NADP^+$

Photoreduction of $NADP^+$ in the light–chloroplast assay was performed as described by Shin et al. [22], with chloroplast fragments depleted of ferredoxin and FNR. FNR and the two flavodoxins from *Chlorella* were used as mediators. The assay mixture contained, in a volume of 1 ml, 50 mM Tris/HCl, pH 8.2, chloroplast fragments (35 μg/ml of chlorophyll), 0.5 mM $NADP^+$, 10 mM sodium ascorbate, 19 μM 2,6-dichlorophenol–indophenol, and different amounts of FNR and ferredoxin, flavodoxin I or flavodoxin II. The reaction was performed at 25 °C in a Hewlett–Packard diode array spectrophotometer, provided with a cuvette holder made from transparent material (methacrylate) and built in our laboratory. The reaction was started by illuminating the reaction mixture with a halogen lamp, and increments in A_{340} produced by NADPH formation were recorded.

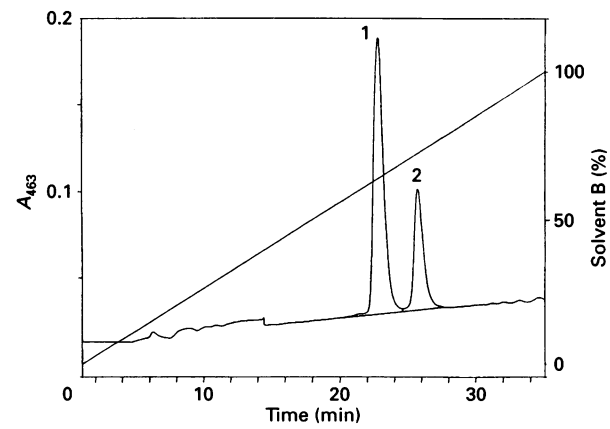

Figure 1 H.p.l.c. elution profile of the two flavodoxins in a Hydropore-5-SAX column

Solvent A was 3 M $(NH_4)_2SO_4$ in 20 mM potassium phosphate, pH 7, and solvent B was 20 mM potassium phosphate, pH 7. A linear gradient from 0 to 100% of solvent B was performed over 35 min. Absorbance was recorded at 463 nm. Peak 1, with a retention time of 22.7 min, corresponds to flavodoxin II (20 kDa), and peak 2 (25.7 min) is flavodoxin I (22 kDa).

RESULTS AND DISCUSSION

Flavodoxin from *Chl. fusca* was described many years ago [4] as a single protein belonging to the large group of flavodoxins and tentatively composed of two subunits of about 11 kDa each. Our flavodoxin preparations, however, although apparently pure according to spectral characteristics, show two different bands on SDS/PAGE, one corresponding to a molecule of molecular mass 22 kDa (flavodoxin I) and a second one of 20 kDa (flavodoxin II). It was very difficult to separate the two proteins as two overlapping peaks were usually obtained. They were, nevertheless, very easily isolated by using anion-exchange h.p.l.c., operating in a reverse gradient (see the Materials and methods section). A typical chromatogram is shown in Figure 1, with flavodoxin II in the first peak and flavodoxin I in the second. SDS/PAGE revealed the flavodoxin composition of the different fractions from the purification (Figure 2). The relative amounts of the flavodoxins are variable in each preparation. Western blots of crude extracts from axenic cultures of cells showed the presence of the two different flavodoxins only when iron was absent from the culture medium (Figure 3). No flavodoxin was found when iron was present, which indicates that small amounts of flavodoxin are not constitutive as reported

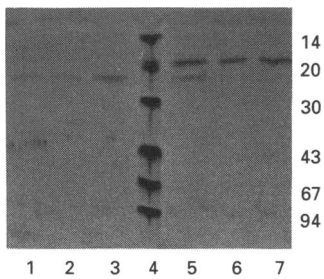

Figure 2 SDS/PAGE of the eluted fractions shown in Figure 1

Lanes 1–3, fractions corresponding to peak 2 (flavodoxin I); lane 4, molecular-mass standards (values indicated on the left in kDa); lane 5, mixture of the two flavodoxins, before h.p.l.c.; lanes 6 and 7, fractions corresponding to peak 1 (flavodoxin II).

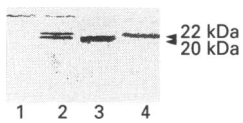

Figure 3 Western blot using antibodies against flavodoxin I and flavodoxin II simultaneously

Lane 1, crude extract from cells cultured in axenic and iron-replete conditions; lane 2, crude extract from cells grown in iron-deficient conditions and maintained carefully in axenic conditions; lane 3, purified flavodoxin II; lane 4, purified flavodoxin I.

previously [4], but are probably induced by some unidentified stress in the batch cultures. The simultaneous presence of the two flavodoxins in crude extracts from axenic cultures excludes the possibility that the 20 kDa flavodoxin is a contaminant organism. In the blot shown in Figure 3, an extra band of around 30 kDa is observed in crude extracts from cells grown in either the presence or absence of iron. Different interpretations can be suggested for this cross-reactive protein, and further work is required to clarify its nature.

Treatment of the pure 22 kDa protein with denaturing agents, such as 8 M urea, 6 M guanidinium chloride or thiol reagents,

Chlorella fusca I	A L G I Y Y S T A T G K T Q E V A D L I K D A L G X A
Chlorella fusca II	Q T G L F Y S T D T G K T E E V A D L I Q W A M G T
Chondrus	K I G I F F S T S T G N T E V A D F I G K T L G A K
Porphyra	G I A V I Y A S T G

Figure 4 Comparison of N-terminal amino acid sequence of flavodoxins from *Chl. fusca* with those of other eukaryotic flavodoxins

The sequences for *Chondrus* and *Porphyra* were taken from [23] and [6] respectively. ● highlights identity in all four sequences, ★ highlights identity in three and ▼ highlights identity in the *Chlorella* sequences.

and also carboxymethylation, did not cause dissociation of the protein into two subunits. The 22 kDa flavodoxin from *Chlorella* is therefore a single polypeptide chain rather than a dimer of two subunits as previously proposed [4].

Purified flavodoxins [flavodoxin I (22 kDa) and flavodoxin II (20 kDa)] from cells grown in the presence of 0.18 μM iron were characterized using several physicochemical methods. The purified proteins had typical flavodoxin u.v.–visible absorption spectra, with slight differences in their maxima (462 nm for flavodoxin I and 464 nm for flavodoxin II) and exhibited spectral ratios of A_{273}/A_{462} of 5.4 for flavodoxin I and A_{272}/A_{464} of 6 for flavodoxin II. N-terminal sequences were determined and are compared in Figure 4 with flavodoxin N-terminal sequences from other eukaryotic sources. The two *Chlorella* flavodoxins have different, but quite homologous, N-terminal sequences, which probably indicates that the lower-molecular-mass flavodoxin is not a degradation product. Flavodoxins I and II exhibit a high degree of homology with one another and, to a lesser extent, with flavodoxin from *Chondrus* [23]. They also show a remarkably higher sequence similarity to cyanobacterial flavodoxins than to flavodoxins from other bacteria [21,24]. Also, their helix-1 segments exhibit a net charge of -1 and -2 for flavodoxin I and flavodoxin II respectively. The charges are more similar to those found for cyanobacteria (net charge negative) than those found for nitrogen-fixing bacteria (net charge positive). It has been suggested by Drummond [25] that the proximity of this helix segment to the FMN coenzyme and the charge distribution in it may have functional significance in the interaction of flavodoxins with other redox proteins. No cross-reactivity was found between the antibodies prepared against *Chlorella* flavodoxins and *Anabaena* PCC 7119 flavodoxin, nor did antibodies prepared against *Anabaena* flavodoxin cross-react with the proteins from *Chlorella*. Antibodies raised against flavodoxin I recognized both flavodoxin I and flavodoxin II, but flavodoxin II antibodies only cross-reacted with flavodoxin II.

H.p.l.c. analysis of flavodoxin II showed that 1 mol of FMN was bound per mol of apoprotein. The presence of this form of flavin is a general feature of all flavodoxins described and was also found in flavodoxin I [4].

Isoelectric focusing of the two forms was performed using a Pharmacia Phast system and both gave low pI (3.7 for flavodoxin I and 3.6 for flavodoxin II), as is characteristic of flavodoxins and ferredoxins (not shown).

It was of interest to determine whether *Chlorella* flavodoxins contain covalent or labile phosphate as described for *A. vinelandii* (strain O.P) flavodoxin [26]. The phosphorus content was determined for both purified flavodoxins: for flavodoxin I, 1 mol of phosphorus/mol of holoflavodoxin was found, corresponding to the FMN residue; no phosphorus was found covalently bound in the apoprotein, indicating that, under the present conditions, phosphate does not modify the flavodoxin. Similar results were obtained for flavodoxin II.

The oxidation–reduction properties of the FMN in flavodoxins

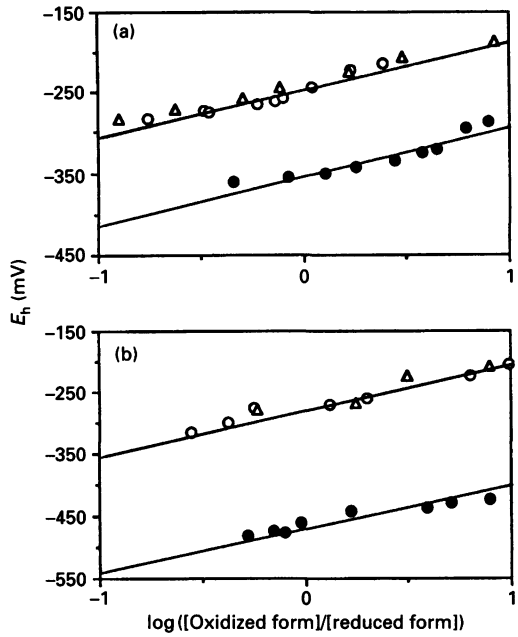

Figure 5 Nernst plot of oxidation–reduction potential data of *Chlorella* flavodoxins at pH 8

Semiquinone formation was measured at 462 or 464 nm and 590 nm. (**a**) Flavodoxin I; (**b**) flavodoxin II. Oxidized/semiquinone redox couple reductive (△) and oxidative (○) titration and semiquinone/hydroquinone redox couple reductive titration (●) are shown.

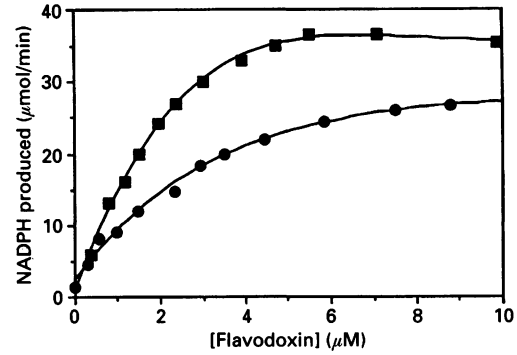

Figure 6 Flavodoxin I- (●) or flavodoxin II- (■) catalysed NADP⁺ photoreduction by FNR from *Chlorella*, using thylakoids from washed spinach chloroplasts (35 μg of chlorophyll/ml) freed of ferredoxin and FNR

FNR was added at a concentration of 11.9 nM, and the flavodoxin concentrations were as indicated.

differ considerably. Redox potentials of *Chlorella* flavodoxin I were measured for both the oxidized/semiquinone (E_2) and semiquinone/hydroquinone (E_1) couples. Nernst plots are shown in Figure 5. The redox potentials were determined at pH 8, the pH of the chloroplast stroma during CO_2 assimilation. The values obtained for the two one-electron reactions in which flavodoxin is involved were −240 mV for E_2 and −350 mV for E_1 in flavodoxin I and −270 mV for E_2 and −458 mV for E_1 in flavodoxin II. If flavodoxin replaces ferredoxin in the whole range of reactions in which the latter participates, it is generally accepted that only the midpoint potential of the semiquinone/hydroquinone couple is negative enough to enable this replacement, and different roles may be assumed for the two flavodoxins. The E_2 value found for *Chlorella* flavodoxin I is considerably more electropositive than that reported for cyanobacterial flavodoxins [21], and very similar to that described for *Chondrus* (−370 mV) [27]. Such an electropositive semiquinone/hydroquinone potential suggests that this flavodoxin may not effectively replace ferredoxin in the photoreduction of NADP⁺. As suggested for *Chondrus* flavodoxin [27], *Chlorella* flavodoxin I is expected to be less active in the photosynthetic electron transfer than ferredoxin or flavodoxin from other sources. Zumft and Spiller [4] reported that flavodoxin from *Chlorella* shows only 60% of the activity observed for ferredoxin in the NADP⁺ reduction catalysed by spinach chloroplasts. In contrast, flavodoxin II fulfils the redox requirements for photosynthetic electron transport, with a midpoint redox potential for the semiquinone/hydroquinone couple of −458 mV. Therefore their respective redox properties indicate that the metabolic roles of the two flavodoxins are different, as in the case of *A. chroococcum*, which has only one flavodoxin able to function as an electron donor to nitrogenase [12].

In order to clarify the possible roles of the two flavodoxins, their efficiency in NADP⁺ photoreduction was assayed. Active chloroplast fragments from spinach, depleted of ferredoxin and FNR, were used. Figure 6 shows the flavodoxin-mediated electron flow from photosystem I to NADP⁺, expressed as NADPH production, in the light and in the presence of *Chl. fusca* FNR and an excess of NADP⁺. As expected from the redox data described above, flavodoxin II is more active than flavodoxin I in NADP⁺ photoreduction. The K_m values were calculated using Eadie–Hofstee plots (not shown) and found to be 1.5 μM for flavodoxin I and 2.65 μM for flavodoxin II. When ferredoxin was used as a reference, the K_m obtained was 4.5 μM. Very similar K_m values and rates of reduction to those for flavodoxin II were found when flavodoxin and ferredoxin from the cyanobacterium *Anabaena* were used [16,28]. These results suggest that, in *Chl. fusca* grown under iron-deficient conditions, flavodoxin II would efficiently replace ferredoxin in the photosynthetic electron flow, whereas flavodoxin I, with lower rates of NADP⁺ reduction, may also replace ferredoxin or play a different undetermined role.

We conclude that under iron-deficient conditions, *Chl. fusca* induces the synthesis of two flavodoxins with similar, but clearly different, amino acid sequences. We have also found a possible difference in the physiological roles of the two forms. The smaller form of flavodoxin is not a degradation product of flavodoxin I, because all the purifications were performed in the presence of protease inhibitors. Also, the cultures were axenic, and no evidence has been found for *in vitro* transformation of flavodoxin I to flavodoxin II. The flavodoxin described by Zumft and Spiller [4] corresponds to our flavodoxin I. The *in vivo* transformation of flavodoxin I to flavodoxin II is not likely to occur because their immunological differences and their N-terminal sequences strongly suggest that they are genetically different. This was suggested previously by Bagby et al. [12] for the two forms of flavodoxin described in *A. chroococcum*. Further confirmation of the presence of one or two genes will be necessary using molecular-biology techniques. The heterogeneity of the *Chlorella* strain used is another possible explanation for the presence of two different flavodoxin isoforms, even though we have not detected such differences in our cells. The presence of endosymbiotic bacteria could also explain the flavodoxin II in our preparations, but the homology between the N-terminal residues suggests a eukaryotic origin of flavodoxin II from a species phylogenetically very close to *Chlorella*, and we have not detected

any contamination in our cultures. This was confirmed by Western-blot analysis of crude extracts from axenic cells, showing the simultaneous presence of the two flavodoxins. The newly described flavoredoxin [13] could be another case of a flavodoxin-like protein involved in other physiological roles with different molecular and redox characteristics similar to the flavodoxin II described here.

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