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ISOLATION AND PROPERTIES OF A FERREDOXIN FROM *ANABAENA FLOS-AQUAE*

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Abstract—Ferredoxin was isolated from the blue–green alga *Anabaena flos-aquae*. Its homogeneity was shown by conventional and SDS-polyacrylamide gel electrophoresis, and isoelectric focusing on polyacrylamide gel columns, the latter indicating a pI at *ca* pH 3·7. The absorption spectrum had, in the oxidized state, maxima at 462, 421, 327 and 276 nm, with a shoulder at 284 nm, a spectrum characteristic of plant-type ferredoxins. The 421 : 276 nm absorbance ratio was typically 0·49. The ferredoxin effectively mediated the photoreduction of $NADP^+$ by barley chloroplasts depleted of native ferredoxin. The MW obtained by sedimentation–equilibrium and sedimentation velocity–diffusion coefficient studies was *ca* 12000 daltons, a value somewhat higher than suggested by amino acid composition data. The ferredoxin contained 2Fe and 2S per molecule.

INTRODUCTION

The iron–sulphur proteins known as ferredoxins play a major role in plant and bacterial photosynthesis [1]. They are small proteins with MWs of *ca* 6000 or 11000 daltons, possessing Fe and labile S in equimolar amounts of 2, 4 or 8 per molecule, depending on the source. They exhibit a negative redox potential and an EPR signal ($g = 1.94$) in the reduced state [2]. The structure and properties of ferredoxins have been recently summarized [1–4].

Several ferredoxins from higher plants have been well characterized; they have MWs of *ca* 11000, contain two atoms each of Fe and labile sulphide, and accept one electron on reduction. The ferredoxins of photosynthetic bacteria are less well characterized, though some, like those of Clostridia and Peptostreptococci (anaerobic fermentative bacteria), appear to contain eight atoms each of Fe and labile sulphide, and accept two electrons on reduction [2].

The ferredoxins of blue–green algae are of interest since their study might give some insight into the biochemical evolution of photosynthesis [5]. These algae may represent an intermediate stage in evolution between the anaerobic photosynthetic bacteria and green plants, as evidenced by localization of their photosynthetic pigments in lamellae distributed throughout the cytoplasm and not organized in discrete organelles. Ferredoxins from several members of the Cyanophyceae have now been isolated and partially characterized [6–19]. The present report describes the isolation and some properties of a ferredoxin from the blue–green alga *Anabaena flos-aquae* which has not been previously studied.

RESULTS AND DISCUSSION

Apart from the difficulty of removing contaminating nucleic acid, *Anabaena flos-aquae* ferredoxin was readily isolated in a high state of purity in yields of *ca* 20 mg from 50 g wet wt cells (Table 1). On conventional polyacrylamide gel electrophoresis the ferredoxin migrated as a reddish–brown band at the anion front. A single band was visible in the corresponding position after staining

Table 1. Purification of ferredoxin from *Anabaena flos-aquae*

	Volume (ml)	Protein (mg)	Protein concn (mg/ml)	A420:276
Acetone powder extraction	320	1920	6·0	
Streptomycin sulphate treatment	350	1050	3·0	(0·05)
1st DEAE-cellulose chromatography	40	78	2·0	(0·05)
2nd DEAE-cellulose chromatography	9	63	7·0	(0·13)
Sephadex G-50	30	48	1·6	0·29
3rd* and 4th DEAE-cellulose chromatography	5·5	21	3·8	0·49

* Fractions with A420:276 greater than 0·35 were collected. The starting material was 49 g wet wt cells, yielding 20 g acetone powder.

with Coomassie blue. Electrophoresis in the presence of 0·1% SDS similarly confirmed the homogeneity of the preparation, though under the conditions used in these studies lower MW contaminants would not be detected. In some preparations a minor component was present, but this formed a very small proportion of total protein. In isoelectric focusing studies the ferredoxin concentrated as a well-defined reddish–brown band at a position corresponding to pI 3·7, though staining with Coomassie blue also revealed the presence in some preparations of a faint band at pI 4·0. The only other pI reported for a ferredoxin, that from *Cl. pasteurianum* [20], is also at pH 3·7, while the pI for *Nostoc* sp.MAC ferredoxin is at *ca* pH 3·4 (Hutson and Rogers, unpublished).

The visible and UV absorption spectrum of *Anabaena flos-aquae* ferredoxin showed absorption maxima at 276, (284), 327, 421 and 462 nm, in good agreement with reports for ferredoxins isolated from other blue–green algae [7–9, 11–13, 16, 18, 19]. The relative absorbances for the main peaks were 1·00, 0·70, 0·50 and 0·47, respectively. The ratio of A at higher wavelengths to that at 276 nm in typical preparations were thus slightly lower than some quoted values in the literature. The shoulder at *ca* 284 nm has also been noted for *Anabaena variabilis* [13] and *Phormidium foveolarum* [19] ferredoxins, and is evident in the published spectrum for *Synechococcus lividus* ferredoxin [16].

The activity of the ferredoxin was measured by its ability to catalyse $NADP^+$ photoreduction in barley chloroplasts depleted of native ferredoxin. With addition of 0·1, 0·2 and 0·3 mg ferredoxin to the standard assay rates of $NADP^+$ photoreduction, measured over the first 30 sec illumination, were 87, 101 and 111 μmol $NADP^+$ reduced/hr/mg chlorophyll, respectively. No $NADP^+$ photoreduction was observed if supplemented ferredoxin was omitted, or if saturating (0·3 mg) levels of ferredoxin were present but the chloroplasts omitted. This activity is somewhat higher than ferredoxin from *Anacystis nidulans* [11] and *Nostoc* sp [8] though higher values can be calculated from data given for *Anabaena variabilis* ferredoxin [13]. Although not measured in the present study it is assumed that *Anabaena flos-aquae* ferredoxin, like other blue–green algal ferredoxins, carries out a transfer of 1 electron [10, 11, 18] and has a redox potential of *ca* − 400 mV [8].

Analyses of the ferredoxin for Fe gave values of 1·77, 1·80, 1·79 and 1·74 atoms per molecule, while assays of labile sulphide gave corresponding values of 1·70, 1·87, 1·72 and 1·59 atoms, based on a MW of 12000. Thus *Anabaena flos-aquae* ferredoxin possesses 2 Fe and 2 labile sulphide, in common, apart from one report [12], with other blue–green algal ferredoxins [16–19].

The amino acid composition, based on three analyses on separate occasions, is given in Table 2. The samples were not oxidized before analysis; the value for half-cystine has therefore to be assumed, since large losses occur on hydrolysis. We attempted to estimate cysteine by titration with 5,5′ dithio*bis* (2-nitrobenzoic acid), but this was only successful after treatment of the ferredoxin with urea; even then only one, or possibly two; cysteines could be identified. A value of 5 is therefore given based on the free-SH demonstrated by chemical analysis, and the four cysteines which must be involved at the active site in a 2 Fe:2S ferredoxin, and which are unreactive with the reagent. The amino acid composition is similar to those reported for some other blue–green algae [12, 16, 18, 19] (Table 2) and enables characteristic differences compared to ferredoxins from photosynthetic bacteria [21] to be identified. Thus three or more lysine, one histidine, and one arginine are usually present; there also appears to be more leucine, but cysteine is less. In common with all ferredoxins there is a preponderance of acidic over basic residues. The total of 95 amino acids, with 2 Fe + 2S, gives a minimum value for MW of 10454.

The partial sp. vol. of the protein derived from the amino acid composition is 0·72, and this value

Table 2. Amino acid composition of blue-green algal ferredoxins

Amino acid	*Anacystis nidulans* [12]	*Synechococcus lividus* [16]	*Phormidium foveolarum* [19]	*Microcystis flos-aquae* [18]	*Nostoc.* sp. MAC*	*Anabaena flos-aquae*
Lysine	3	4	2	3	5	3
Histidine	1	1	1	1	1	1
Arginine	1	3	1	1	1	1
Tryptophan	0†	0†	0	—	—	0†
Aspartic acid	15	11	13/14	13	12/13	13
Threonine	12	6	10	7	10	8
Serine	7	4	5/6	6	6	7
Glutamic acid	11	17	14	13	12/13	14
Proline	3	5	4	4	3	3
Glycine	6	6	6	12	6	6
Alanine	12	6	8	9	6	8
Half-cystine	6	5	4‡	5	5	5‡
Valine	9	7	5	4	7	7
Methionine	0	0	0	1	0	0
Isoleucine	5	4	7	6	6	5
Leucine	7	10	8	9	8/9	7
Tyrosine	5	4	5	3	5	4
Phenylalanine	2	3	2	1	1	3

* K. G. Hutson and L. J. Rogers (unpublished).
† Estimated spectrophotometrically.
‡ Assumed.
– Not determined.

was used in the calculation of MW from ultracentrifuge data. Low speed sedimentation equilibrium experiments yielded MWs of 10000, 12000 and 12500, and meniscus depletion sedimentation equilibrium experiments gave values of 12000 and 13000. A MW of 12500 was calculated by the Svedberg equation from experimentally determined values for $s_{20,w}$ of $1{\cdot}5 \times 10^{-13}$ sec, and $D_{20,w}$ of $10{\cdot}4 \times 10^{-7}$ cm^2 sec^{-1}. The average MW from these ultracentrifuge studies is 12000, a somewhat higher value than the 10500 given by calculation from amino acid composition. For proteins of this low MW the values derived by physicochemical methods are useful in defining the MW range, but in our view may not be as accurate as values then derived from amino acid composition. A MW of *ca* 11000 has been reported [8, 12, 16, 18, 19] for ferredoxins from a number of blue-green algae. MW determination on an aged preparation yielded a value near 20000; possibly under these conditions ferredoxin may undergo aggregation [22]. A MW of *ca* 20000 has been reported for a ferredoxin from *Azotobacter vinelandii* [23].

Anabaena flos-aquae ferredoxin is clearly similar in properties to the ferredoxins of the eukaryotic algae and higher plants, as have been the ferredoxins from other blue-green algae.

EXPERIMENTAL

Growth of Anabaena flos-aquae. (Lyng.) Breb. Tischer's strain was obtained from the Cambridge Culture Collection (strain no. 1403/13), The Botany School, Cambridge, England. It was grown in 5 × 10 l. culture medium by methods described in [24]. Before the algae were harvested individual flasks were tested for purity of cultures by standard procedures.

Purification. Me_2CO-dried cells (*ca* 20 g) were suspended in 300 ml of 15 mM Tris–HCl. This and all subsequent buffers, unless otherwise stated, were at pH 7·7 at 4°. After 2 hr at room temp. the suspension was centrifuged at 40000 *g* for 60 min at 4°. All subsequent manipulations were carried out at 4°. The supernatant was collected and nucleic acid precipitated by treatment with streptomycin sulphate (2·5 mg/mg nucleic acid as estimated by A260:280) and removed by centrifugation at 48000 *g* for 15 min at 4°. The supernatant was brought to 0·15 M with regard to Tris and applied to a 10 × 4 cm dia DEAE-cellulose (Whatman DE-52) column, which had been equilibrated with the Tris–HCl buffer. Ferredoxin was adsorbed on the column which was washed with 1·5 l. of 0·15 M Tris–HCl in 0·1 M NaCl (0·22 M Cl^-). The brownish-red ferredoxin band was then eluted by 0·3 M Tris–HCl in 0·55 M NaCl (0·8 M Cl^-), diluted × 10 with H_2O, and loaded onto a second DEAE-cellulose column (15 × 2 cm dia) previously equilibrated with 0·15 M Tris–HCl in 0·1 M NaCl (0·22 M Cl^-). The column was washed with 500 ml of the Tris–HCl buffer, and ferredoxin then eluted by 0·3 M Tris–HCl in 0·55 M NaCl (0·8 M Cl^-). Eluted ferredoxin was applied to a Sephadex G-50 column (50 × 3 cm dia) previously equilibrated with 0·15 M Tris–HCl in 0·2 M NaCl (0·32 M Cl^-). The column was developed with the same soln, the ferredoxin emerging in *ca* 30 ml vol. This eluate was dialysed against 0·15 M Tris–HCl in 0·2 M NaCl for 3 hr before loading on to a DEAE-cellulose column (20 × 2·5 cm dia) equilibrated with the same buffer and developed with the same soln at 40 ml/hr. Ferredoxin was eluted in the 110–220 ml fraction. The fractions with A420:276 greater than 0·35 were bulked,

diluted with 5 vol. of H_2O, and concentrated on a small DEAE-cellulose column (6 × 1 cm dia) previously equilibrated with 0·15 M Tris–HCl. The ferredoxin was eluted by 0·3 M Tris–HCl in 0·55 M NaCl (0·8 M Cl^-) at a flow rate of 32 ml/hr. Finally the ferredoxin, now in *ca* 6 ml buffer, was dialysed for 3 hr against 0·15 M Tris–HCl. The yield of ferredoxin was *ca* 20 mg, and negligible quantities of nucleic acid were present.

Assay of protein and ferredoxin. During the early stages of purification protein and nucleic acid were estimated from A260:280; in the later stages protein was estimated by the Folin phenol reagent [25]. The A420:276 ratio was taken as an index of ferredoxin content.

Activity determination. Ferredoxin activity was measured by $NADP^+$ photoreduction with barley chloroplasts, prepared as described elsewhere, [26] and depleted of native ferredoxin. In assays each 1 cm light-path cuvette contained in μmol–Tris buffer (pH 8), 45; NaCl, 60; $MgCl_2$, 12; KH_2PO_4, 5; $NADP^+$, 0·485; the required amount of ferredoxin; and chloroplast suspension equivalent to *ca* 30 μg chlorophyll; in a total vol. of 3 ml. Photoreduction of $NADP^+$ was observed by measuring the increase in A at 340 nm on illumination with saturating light, after 30 sec and 1 min illumination. Over this time interval reduction of $NADP^+$ proceeded linearly. Chlorophyll was determined as chlorophyll (*a* + *b*) [27].

Iron, labile sulphide and free cysteine content. Iron content was determined by the method in [28] as modified in [29]. Labile sulphide was determined by the method in [30] as modified in [31] using *N,N'* dimethyl-*p*-phenylenediamine reagent. The blue colour developing in the presence of labile sulphide was followed by increase in A at 670 nm. For reproducible results it was necessary to carry out the estimation in small tightly sealed tubes so that the vol. of the dead-space in the tube was minimized. The sulphide content of the ferredoxin was calculated by reference to A670 given by standard solns of Na_2S treated with the reagents. Cysteine sulphur in the native ferredoxin was estimated by titration with 5,5' dithio*bis* (2-nitrobenzoic acid) by the method of [32]. It was necessary for the ferredoxin to be brought to 1 M in respect to urea by 15 hr dialysis, or be treated with 6 M urea, before reactivity of the ferredoxin could be demonstrated.

Electrophoresis. Polyacrylamide disc-gel electrophoresis was carried out in 10% acrylamide, 0·12% *N,N'*-methylene *bis*acrylamide gels, using 0·15 M Tris–HCl (pH 7·7) as electrolyte. Electrophoresis in SDS-containing polyacrylamide gels was carried out on 10% acrylamide, 0·025% *N,N'*-methylene *bis*acrylamide gels. The gel and electrolyte, 0·2 M KH_2PO_4 (pH 7), contained 0·1% SDS. The protein sample containing 50–100 μg protein in 80 μl 10% sucrose was directly layered on to the surface of the separation gel below the electrolyte. In both cases, following electrophoresis at 14 mA per tube for 3–5 hr, gels were fixed 15 hr in TCA MeOH–H_2O (2:9:9). Gels were stained in 0·2% Coomassie blue in HOAc–EtOH–H_2O (10:45:45) for 15 hr, and destained in 7% HOAc until the blue protein-containing bands were clearly visible against a clear background. Isoelectric focusing was carried out on 6% acrylamide, 0·008% *N,N'*-methylene *bis*acrylamide gels. The gels contained 1% (v/v) ampholine mixture (pH range 3–10) and the pH gradient preformed by passing a current of 1 mA per tube for 1 hr, the electrolyte chambers containing 0·4% (w/v) ethanolamine (cathode) and 0·2% (w/v) H_2SO_4 (anode). During this preliminary pH-gradient establishment gels were overlaid with 0·1 ml 5% sucrose in 5 mM KH_2PO_4 (pH 7·5) containing 1% ampholine mixture. For isoelectric focusing liquid was decanted from the top of the gels and 50–100 μg ferredoxin in 10% sucrose applied, and overlaid with 0·4% ethanolamine to the required vol. in the cathode vessel. Electrophoresis was carried out for 3·5–4·0 hr at 4° with 1 mA per tube (maximum 500 V). Gels were then removed and protein fixed by immersion in 6% TCA for 24 hr. The gels were further washed for 24 hr with several 150 ml vol. of 6% TCA, to remove ampholines, before staining in 1% amido black in 7% HOAc for 1 hr, or 0·2% Coomassie blue in HOAc: EtOH: H_2O (10:45:45) for 15 hr. Gels were destained by repeated washing with 7% HOAc. The pH gradient along the gel was determined by sectioning a gel (not containing protein) immediately after the electrophoretic run, and leaving 5 mm sections in tubes containing 3 ml deionised H_2O for 2–3 hr. The pH of the H_2O in each tube was then measured.

Ultracentrifuge methods. Sedimentation coefficients were determined by established procedure [33] and were corrected using density and viscosity data obtained using a sp. gr. bottle and Ostwald viscometer, respectively. For sedimentation coefficient studies the centrifuge was operated at 59 780 rpm with the temp maintained at 20°. Photographs were taken at 16 min intervals. The diffusion coefficient was obtained in the ultracentrifuge using a synthetic boundary cell to form a sharp interface between dialysate and soln. Photographs were taken of the diffusing boundary using Schlieren optics and the diffusion coefficient was calculated by plotting $A^2/(H^2 2{\cdot}14^2)$ vs time (*A*, area; *H*, maximum height of the peak; 2·14, cell to photographic plate magnification). The meniscus depletion (high-speed) equilibrium method [34] and a low speed sedimentation equilibrium method [35] were used to determine MW directly. The partial sp. vol. of the ferredoxin calculated from amino acid composition was 0·72.

Amino acid composition. Samples were hydrolysed for 24 hr in 6 M HCl in O_2-free sealed ampoules, and analyses were performed on a single column autoanalyser at the Macromolecular Analysis Centre, University of Birmingham. Tryptophan was estimated in native ferredoxin by spectrophotometric techniques, [36, 37] and free cysteine by a chemical method [32].

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