Phytochemistry, 1981. Vol. 20, pp. 383–387. © Pergamon Press Ltd. Printed in England. 0031-9422/81/030383-05 $02.00/0

FLAVODOXIN FROM THE BLUE-GREEN ALGA *NOSTOC* STRAIN MAC

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(*Revised received* 2 *June* 1980)

Key Word Index—*Nostoc* strain MAC; Cyanophyceae; blue-green alga; flavodoxin.

Abstract—A flavodoxin was isolated from the blue-green alga *Nostoc* strain MAC grown photoautotrophically or chemoheterotrophically in iron-deficient medium. *In vitro*, the flavodoxin would support $NADP^+$ photoreduction by photosynthetic membranes, pyruvate oxidation by the phosphoroclastic system of *Clostridium pasteurianum*, and electron transfer to *Cl. pasteurianum* hydrogenase. In its oxidized form, the flavodoxin had absorbance maxima at 274 sh283 sh293, 376 sh432 and 466 sh488 nm. Reduction by dithionite proceeded via a neutral, blue semiquinone radical. The flavodoxin contained 1 mol of FMN per mol of protein and the amino acid composition showed a predominance of acidic residues; cysteine was apparently absent. A minimum MW of *ca* 22 000 derived from these data was confirmed by electrophoresis on SDS-polyacrylamide gels and by ultracentrifugal analysis. This flavodoxin thus belongs to the higher MW group of these low potential electron transfer proteins.

INTRODUCTION

Flavodoxins have been isolated from a variety of organisms, from anaerobic fermentative bacteria to eukaryotic algae. They appear to fall into two groups, one having MW *ca* 15 000 and the other *ca* 22 000 [1]. The flavodoxins isolated from obligate and facultative anaerobic bacteria make up the first group with the exception of that from the photosynthetic bacterium *Rhodospirillum rubrum*. This flavodoxin belongs to the second group which includes those from *Azotobacter vinelandii*, the blue-green algae (cyanobacteria) *Anacystis nidulans* and *Synechococcus lividus*, the green alga *Chlorella fusca* and the red alga *Chondrus crispus* [2, 3]. Flavodoxins are thus not confined to prokaryotic organisms. All the flavodoxins isolated so far contain FMN as the redox active component and the redox potential of the hydroquinone/semiquinone couple is very low [2], similar to that of ferredoxin [4]. Ferredoxins, though smaller than flavodoxins, also fall into two distinct MW groups (6000 and 11 000). Flavodoxin has been shown to be capable of replacing ferredoxin in a number of biological reactions [2].

The blue-green alga *Nostoc* strain MAC normally synthesizes two soluble ferredoxins [5] which have been isolated and extensively characterized [6]. Like other algal ferredoxins, they are of the plant type, containing a single 2Fe–2S active centre. In response to iron deficiency, *Nostoc* strain MAC produces a flavodoxin while amounts of both ferredoxins decrease [7] which is similar to the situation in some other organisms such as *Clostridium pasteurianum* [8] and *A. nidulans* [9]. The biological activities of the flavodoxin from *Nostoc* strain MAC, compared to those of the two plant-type ferredoxins which occur [6], are of interest, as are its structural properties since flavodoxin from another blue-green alga, *A. nidulans*, possessed properties similar to those of flavodoxins from the eukaryotic algae *C. crispus* [3] and *Ch. fusca* [2]. It is thus possible that the prokaryotic blue-green algae, together with the red and green algae, represent intermediate stages in evolution from the anaerobic photosynthetic bacteria to green plants. The present report describes the isolation and some properties of the flavodoxin from *Nostoc* strain MAC.

The other blue-green algae from which flavodoxin has been isolated, *Anacystis nidulans* and *Synechococcus lividus*, are both unicellular organisms in the genus *Synechococcus*. In contrast, *Nostoc* strain MAC is a filamentous blue-green alga, originally isolated from the coralloid roots of the cycad *Macrozamia lucida*, and provisionally assigned to the genus *Nostoc*.

RESULTS AND DISCUSSION

Extracts of *Nostoc* strain MAC grown photoautotrophically or chemoheterotrophically in normal medium produce two ferredoxins [5]. Iron-deficient cultures also produce a flavodoxin.

A typical elution profile for separation of these electron transfer proteins on DEAE-cellulose has been given in Fig. 1c of Hutber *et al.* [7]; (ferredoxin I and II in that legend should be transposed). A significant amount of ferredoxin I was present in cell extracts, but only a trace of ferredoxin II. After several subcultures of the organism into iron-deficient medium, mass cultures still yielded both ferredoxins, suggesting that these are not replaced entirely by flavodoxin. In several organisms, flavodoxin synthesis occurs during growth in media low in iron [2] but flavodoxins from *Escherichia coli* [10], *Az. vinelandii* [11] and *C. crispus* [12] appear to be normal constituents of the cells.

The yield of flavodoxin from photoautotrophic or chemoheterotrophic cultures was on average *ca* 25 mg from 150 g wet *Nostoc* strain MAC cells (Table 1). The absorbance ratio A_{466}/A_{276} which was used to monitor

Table 1. Purification of flavodoxin from *Nostoc* strain MAC. The starting material was 137 g wet wt of cells, yielding 32.4 g of acetone-dried powder

Stage	Total vol. (ml)	Protein concn (mg/ml)	Total protein (mg)	A_{466}/A_{276}
Acetone-dried powder extraction	370	46.0	17000	(0.16)
Supernatant after 60% saturation with $(NH_4)_2SO_4$	390	3.4	1330	0.02
DEAE-cellulose chromatography	4.9	4.7	23.0	0.15
Gel filtration on Sephadex G-150	52	0.45	23.4	0.18
Final preparation after dialysis	4.0	6.0	24.0	0.18

purification was initially very high due to the presence of large amounts of phycobiliprotein. The final value of this was at least 0.18. Values of the absorbance ratio for other flavodoxins vary from 0.17 for *A. nidulans* [13] to 0.23 for *Cl. pasteurianum* [8].

On conventional polyacrylamide gel electrophoresis, the flavodoxin migrated as a single yellow band. After staining for protein, a single band was visible in the corresponding position. On 10% gels the flavodoxin migrated close to the anion front but as the percentage of acrylamide was increased, molecular sieving led to significant retardation. Homogeneity was also indicated by isoelectric focusing studies [14].

The absorption spectrum of the isolated flavodoxin was typical of protein-bound flavin in the fully oxidized form (Fig. 1). The molar absorption coefficient at 466 nm calculated on the basis of a MW of 21000 was 9.5×10^3 l/mol/cm, which is similar to the values for most other flavodoxins [2]. On addition of small aliquots of dithionite in the presence of 3 μM methyl viologen as mediator, the flavodoxin could be reduced anaerobically to a blue semiquinone form. This had a spectrum similar to that of other semi-reduced flavodoxins, and this has been attributed to the neutral radical form of the semiquinone [15]. Under the conditions used, complete reduction of the flavodoxin was not obtained by addition of a stoichiometric amount of dithionite; the hydroquinone may be particularly sensitive to residual traces of oxygen. The spectrum which corresponded to the fully reduced form of the protein was obtained after *ca* 45 min on anaerobic reduction with a 50-fold molar excess of dithionite.

The flavodoxin was initially identified as such by its spectrum and its behaviour on DEAE-cellulose. The prosthetic group, released by heat treatment, was unequivocally identified as FMN by its co-chromatography with authentic FMN. This was confirmed by examining the fluorescence emission of the flavin at pH 2.6 and pH 7.0, conditions under which FMN and FAD behave quite differently. The purified protein did not contain non-haem iron or acid-labile sulphide.

The magnitude of the absorption coefficient at 466 nm indicated the likelihood of one FMN group per protein molecule. Spectrophotometric determination of dissociated flavin yielded a value of 0.79 FMN group per molecule of protein based on a ε_{466nm} of 12.2×10^3 l/mol/cm for FMN, while fluorimetric titration of the apoflavodoxin with dissociated FMN indicated a value of 0.88. Thus like other flavodoxins, *Nostoc* strain MAC flavodoxin contains only one FMN group per protein molecule.

The flavodoxin sedimented in the analytical ultracentrifuge as a single symmetrical peak of sedimentation coefficient 2.32×10^{-13} sec. Observation with both schlieren and Rayleigh interference optics of the diffusion of a

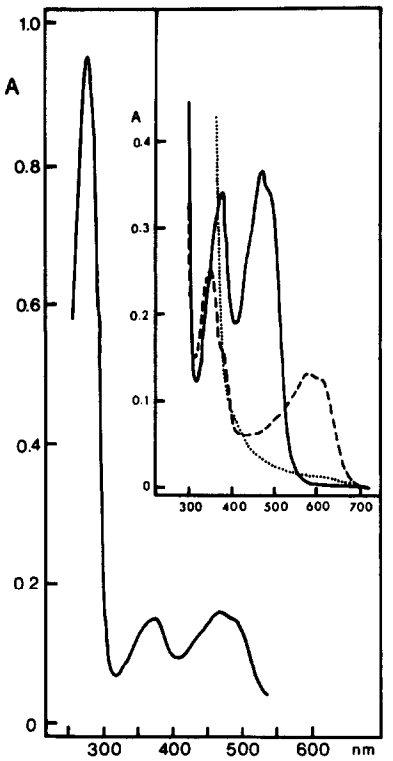

Fig. 1. UV and visible absorption spectra of flavodoxin from *Nostoc* strain MAC. The spectrum is of a solution of 36 nmol of fully oxidized flavodoxin in 3 ml of 0.15 M Tris/HCl, pH 7.4 at 20°. Inset—Flavodoxin, 120 nmol in 3 ml of 0.15 M Tris/HCl (pH 7.4 at 20°) containing 3 μM methyl viologen was titrated under anaerobic conditions with 0.1 mg/ml dithionite solution. The spectra recorded are: ———, before addition of dithionite; – – – –, after addition of *ca* 1 molar equivalent of dithionite; ·····, after addition of a 50-fold molar excess of dithionite. Experiments were carried out in a Thunberg cuvette fitted with a Suba-seal rubber stopper and flushed with pyrogallol-scrubbed argon. Additions of dithionite were by injection through the seal.

Table 2. Properties of *Nostoc* strain MAC flavodoxin. Some results are given as means ± s.d. for *n* experiments

Absorption maxima (oxidized), nm	274 sh283 sh293 376 sh432 466 sh488	
Ratio of absorbance	1.0 0.19 0.20	
Log ε_{466nm}	(3.98)	
Absorption maxima (semiquinone), nm	350 sh380 576 sh606	
Prosthetic group	1 FMN/mol	
Sedimentation coefficient ($s_{20,w}$)	$2.32 \pm 0.08 \times 10^{-13}$ sec; $n = 4$	
Diffusion coefficient ($D_{20,w}$)	$9.00 \pm 0.2 \times 10^{-7}$ cm^2.sec^{-1}; $n = 4$	
MW by Svedberg equation	23 600	
MW by sedimentation equilibrium	$20\,500 \pm 800$; $n = 5$	
MW by amino acid composition	22 000	
MW by SDS-polyacrylamide gel electrophoresis	21 700	
Partial specific volume ($\bar{v}$)	0.73	
Isoelectric point	3.49 ± 0.13; $n = 6$	
Relative activity		
	cf. Ferredoxin I	Ferredoxin II
in $NADP^+$ photoreduction		
(chloroplasts)	1.83	1.08
(*Nostoc* membranes)	1.25	0.78
in phosphoroclastic reaction	0.36	0.40
in H_2 evolution by hydrogenase		
(dithionite)	0.51	0.61
(chloroplasts)	1.59	1.35

boundary formed in the ultracentrifuge using a synthetic boundary cell enabled the diffusion coefficient to be calculated as 9×10^{-7} cm^2/sec; substitution of this value with that of the sedimentation coefficient in the Svedberg equation gave an MW of 23 600. This was slightly higher than the value of 20 500 determined directly by meniscus depletion and low-speed sedimentation equilibrium experiments (Table 2). Neither sedimentation nor diffusion coefficients showed concentration dependence over the range 1–6 mg of protein/ml.

The MW was also determined by electrophoresis on SDS-polyacrylamide gels with standard proteins of known MW. The flavodoxin migrated as a single band at an R_m corresponding to an MW of 21 700. These data and some other properties of the flavodoxin are summarized in Table 2. The flavodoxin from *Nostoc* strain MAC can clearly be placed in the larger MW group of these proteins, together with those from *C. crispus* [3], *Ch. fusca* [16], *Az. vinelandii* [17], *R. rubrum* [18] and *A. nidulans* [13, but see 19]. This contrasts with flavodoxins from anaerobic bacteria such as *Cl. pasteurianum* which have MWs of *ca* 15 000 [8].

The amino acid composition based on four analyses was determined as Asx_{25}, Glx_{28}, Thr_{10}, Ser_{10}, Pro_3, Gly_{20}, Ala_{12}, Cys_0, Val_9, Met_1, Ile_{13}, Leu_{15}, Tyr_7, Phe_{13}, Trp_6, His_2, Lys_{13}, Arg_4. Cysteine and tryptophan were determined separately by spectrophotometric methods. The composition is broadly similar to that of other flavodoxins from both MW groups in containing a large proportion of acidic residues and appreciable amounts of the small non-polar residues glycine and alanine [20]. Unlike the other flavodoxins, no cysteine was detectable by titration with DTNB or PCMB in either native or guanidine hydrochloride-denatured protein. The total of 191 amino acids together with one FMN group gives a minimum MW for *Nostoc* strain MAC flavodoxin of 22 000. The partial specific volume derived from the composition ($\bar{v} = 0.73$ cm^3/g) was used in calculation of the MW from sedimentation equilibrium experiments.

The pI of *ca* 3.5 was the same as that of *C. crispus* flavodoxin, and somewhat higher than that of flavodoxin from *Cl. pasteurianum* which had a pI of 3.15 [14].

Nostoc strain MAC thus responds to iron-deficient culture conditions by synthesizing a flavodoxin which is similar to that from another blue-green alga, *A. nidulans* [13], and those from eukaryotic algae [3, 16]. This was not unexpected, as the two ferredoxins produced by *Nostoc* strain MAC are also of the plant type [5, 6]. Preliminary studies of the amino acid sequence of the flavodoxin (G. N. Hutber, L. J. Rogers, I. Takruri and D. Boulter, unpublished data) indicate the presence of the expected homology with other flavodoxins at the N-terminal end of the molecule [23], though this is less than the homology between flavodoxins of the anaerobic bacteria.

Flavodoxins appear to substitute for ferredoxins in many of the wide range of reactions in which the latter can function as electron carriers [21]. In *A. nidulans*, the other blue-green alga studied in this respect, the flavodoxin was equally or more effective *in vitro* than ferredoxin in supporting $NADP^+$-photoreduction, nitrite reduction, and the phosphoroclastic system [9]. Comparative studies of the activities of the two ferredoxins and flavodoxin from *Nostoc* strain MAC (Table 2) show that the flavodoxin is as active as ferredoxin II in catalysing $NADP^+$-photoreduction by chloroplast preparations (55 μmol $NADP^+$/hr/mg chlorophyll) whereas ferredoxin I is considerably less active. With *Nostoc* membrane

particles the relative activities of the ferredoxins are about the same but the flavodoxin now shows intermediate activity (21 μmol $NADP^+$/hr/mg chlorophyll). The relative activities reflect the redox potentials of the three proteins. The more effective ferredoxin II has a midpoint redox potential of −455 mV [22], whereas ferredoxin I, the predominant ferredoxin *in vivo* has a more positive potential (−350 mV) and so is less suitable to mediate in electron transfer from photosystem I to $NADP^+$. The redox potential of the flavodoxin semiquinone/hydroquinone couple based on the value reported for *A. nidulans* flavodoxin [9], would be similar to that of ferredoxin II. In contrast to the situation with *A. nidulans* the flavodoxin from *Nostoc* strain MAC was *ca* half as active as either ferredoxin in supporting pyruvate oxidation by the phosphoroclastic system of *Cl. pasteurianum* but nevertheless supported a rate of *ca* 7 μmol acetyl-P/hr in a standard assay [6]. It has also proved possible in collaboration with Dr. K. K. Rao and Professor D. O. Hall, Department of Plant Sciences, University of London, King's College, to assess the activities of the proteins in two hydrogenase assays. In supporting electron transfer from dithionite to *Cl. pasteurianum* hydrogenase, *Nostoc* strain MAC flavodoxin supported a rate of 9 μmol H_2 evolved/hr which was again less active than either ferredoxin. However, these relative activities were reversed when electrons were donated from photosystem 1, using an illuminated spinach chloroplast system; here the flavodoxin supported a rate of 21 μmol H_2 evolved/hr/mg chlorophyll. Other than $NADP^+$ photoreduction, the *in vivo* status of *Nostoc* strain MAC with regard to these biochemical activities is uncertain.

EXPERIMENTAL

Nostoc strain MAC was maintained and grown on medium containing 0.5 μM Fe as described previously [7].

Purification of flavodoxin. Flavodoxin was extracted and purified from Me_2CO-dried powders by fractionation with $(NH_4)_2SO_4$, and chromatography on DEAE-cellulose [7]. The flavodoxin was usually over 90% pure at this stage as assessed by polyacrylamide gel electrophoresis. Occasionally, it was contaminated by small amounts of other proteins which were removed by rechromatography on DEAE-cellulose or by gel filtration on a column (90 cm × 2.5 cm) of Sephadex G-150 equilibrated and developed with 0.05 M Tris/HCl, pH 7.8 at 4°. Fractions with A_{466}/A_{276} greater than 0.18 were pooled and concd on a small DEAE-cellulose column before dialysis and immediate use or storage at −20°. The purification of flavodoxin was assessed by the A_{466}/A_{276} ratio and final yield determined by estimation with Folin PhOH reagent using BSA as standard [24].

Analytical PAGE. The technique of ref. [25] was used, without spacer or sample gels. Electrophoresis was carried out at pH 8 on 10, 15, 20 and 25% acrylamide gels; the bis-acrylamide was a constant proportion (3.5%) of the total acrylamide. For detection of protein components in the gels, they were stained with Coomassie Brilliant Blue [26] or Coomassie Brilliant Blue G250 in $HClO_4$ [27, 28]. The pI was determined by electrofocusing over the pH range 2.5–5 on thin layers of polyacrylamide gel, the pH gradient along the gel being measured directly using a surface electrode [14]. The MW of the flavodoxin was determined by its relative migration rate on PAGE in SDS [29] containing 10% (w/v) total acrylamide in relation to the following standards: catalase, 60000; fumarase, 49000; aldolase, 40000; glyceraldehyde 3-phosphate dehydrogenase, 36000; trypsin, 23000; ribonuclease, 13000. After electrophoresis the protein bands were fixed and the SDS leached out by immersion overnight in 10% TCA and MeOH (1:1) before staining with Coomassie Brilliant Blue.

Identification of prosthetic group. The prosthetic group was dissociated from apoprotein in the dark by heating to 80° for 10 min or by treatment with 3% aq. TCA, and identified by co-chromatography with authentic FMN and FAD on cellulose TLC plates in three different solvent systems [30]. The identity of the flavin was confirmed spectrofluorimetrically by comparison with authentic FMN and FAD at pH 2.6 and pH 7 [31, 32]. The amount of prosthetic group released from a known amount of flavodoxin was estimated both spectrophotometrically and spectrofluorimetrically [33].

MW. Ultracentrifuge studies were performed using a Beckman–Spinco Model E analytical ultracentrifuge equipped with both schlieren and Rayleigh interference optics. Operating procedures were as detailed in the manufacturers' instruction manual and standard texts [34]. All expts were at 20° with flavodoxin soln in 0.15 M Tris/HCl, pH 7.4. Sedimentation velocity expts were performed at a speed of 63650 rpm using the schlieren optical system. Diffusion coefficient studies were carried out at 10000 rpm with either a single sector, reservoir-type synthetic boundary cell (schlieren optics) or a double sector synthetic boundary cell (Rayleigh interference optics). For the first of these, the diffusion coefficient was estimated by the conventional area-height analysis [34]; in the latter case by analysis of boundary spreading.

Short column sedimentation equilibrium expts using Rayleigh interference optics were performed with a double sector cell, with flavodoxin soln in one compartment and buffer (dialysate) in the other. Expts by the method of ref. [35] were performed at a speed of 17980 rpm at an initial protein concn of 2 mg/ml. For the method of ref. [36] the speed was 37020 rpm at an initial protein concn of 0.5 mg/ml. The flavodoxin was quite stable for the duration (24–36 hr) of the expts.

The partial specific vol. ($\bar{v}$) was calculated from the amino acid composition by the method of ref. [37].

Amino acid composition. Samples were hydrolysed at 110° for 24 or 72 hr in 6 M HCl in O_2-free sealed ampoules and analyses were performed on a Locarte single column autoanalyser at the Macromolecular Analysis Centre, University of Birmingham, U.K. The amino acids were quantified through a Nova 1220 computer on-line to the analyser. The values of threonine, serine and tyrosine were obtained by extrapolating to zero time and those of valine and isoleucine were of 72 hr hydrolyses. Tryptophan was determined separately by a spectrophotometric method [38] and cysteine by reaction with DTNB [39] and PCMB [40]; in the latter cases the flavodoxin was titrated with reagent and $\Delta A_{412\,nm}$ or $\Delta A_{250\,nm}$, respectively, was followed.

Activity determinations. Assays of $NADP^+$ photoreduction, and pyruvate oxidation by the phosphoroclastic reaction, are given in ref. [41]; hydrogenase assays were as in ref. [42].

Acknowledgement—G.N.H. was in receipt of an SRC studentship.

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