CHARACTERIZATION OF A FLAVODOXIN FROM THE GREEN ALGA CHLORELLA

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Received July 26, 1971

SUMMARY

A new flavoprotein has been isolated and purified to homogeneity from the green alga Chlorella fusca. It has characteristics comparable to those of phytoflavin from the blue-green alga Anacystis nidulans or flavodoxin from various bacteria. Chlorella flavodoxin can replace spinach and Chlorella ferredoxin in photosynthetic NADP reduction of broken chloroplasts. Its molecular weight is 22 000. One mole of FMN is present per mole of protein. The extinction coefficient at 464 nm is 10 000 M^{-1} cm^{-1}. Reduction of the flavoprotein proceeds via a blue flavosemiquinone radical. The amino acid composition reveals a close relationship to bacterial flavodoxins.

Low molecular, low potential flavoproteins as a new class of electron carriers are capable of replacing ferredoxin in its various reactions (1). Smillie was the first to isolate from the blue-green alga Anacystis nidulans a flavoprotein, named phytoflavin that acts instead of ferredoxin in the light-dependent reduction of NADP by chloroplasts (2). Later on closely related flavoproteins, flavodoxins, were otained from various bacteria (1). Recently Vetter and Knappe reported the isolation of a ferredoxin-like substance as well as a flavodoxin from the bacterium Escherichia coli K 12 (3).

The properties and functional role of phytoflavin from Anacystis in a series of photosynthetic reactions have been studied by Bothe (4) and Bothe et al. (5). Since flavodoxins have been found only in procaryotic organisms they seemed to be a unique feature of bacteria and blue-green algae. We wish to re-

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port on the detection of a flavodoxin+ from the eucaryotic green alga Chlorella. Similar investigations with Ankistrodesmus braunii under virtually the same conditions yielded another flavoprotein mediating NADP reduction of broken chloroplasts. Thus an ubiquituous distribution of this electron transfer protein seems to be probable. Some of its properties have been investigated and are compared with those of bacterial flavodoxins, ferredoxin, and phytoflavin from other sources.

MATERIAL AND METHODS

Cells of Chlorella fusca Shihira et Krauss, strain 211-15 (=pyrenoidosa), from the culture collection at Göttingen, were grown as described elsewhere (6). Instead of 8 mg $FeSO_4.2H_2O$/l only 0.03 mg were used to produce iron-deficient conditions. This concentration still allowed a good growth of the organism giving at the same time high yields of the electron carrier. After 12 days the cells were broken and the resulting homogenate was freed from cell particles by high-speed centrifugation. The purification procedure of flavodoxin resembled that of ferredoxins and consisted of chromatography on DEAE-cellulose (DE-52, Whatman), elimination of nucleic acids by precipitation with a neutral 2 % protamine sulfate solution, a second passage through DEAE-cellulose and gelfiltration in Sephadex G-75. This process gave a homogeneous protein as shown by analytical disc electrophoresis in a 7.5% gel at pH 6.0. The specific activity, measured as micromoles NADP reduced/min per mg protein rose from 0.015 in the centrifugation supernatant to 2.83 after the last purification step, resulting in an overall purification of approx. 200-fold. The activity of flavodoxin was assayed like ferredoxin (7). Broken chloroplasts (8) and ferredoxin (9) were prepared from green-house spinach. Chlorella ferredoxin was obtained by a method of Zumft (10). Gel filtration experiments were performed with a Sephadex G-75 column (1.5 x 80 cm) according to Andrews (11). Polyacrylamide electrophoresis in the presence of dodecylsulfate using the normal amount of cross linker followed the method

+Following the suggestions of Bothe, Hemmerich and Sund (5) one of the names should be given up. Despite Smillie's nomenclature we prefer the more relevant name 'flavodoxin'.

of Weber and Osborne (12). FMN was identified by paper chromatography (13); extinction coefficients were determined by dissociation of FMN from the protein by trichloracetic acid (14). Spectral measurements were done in a Zeiss PMQ II or a Beckman DB-GT spectrophotometer.

RESULTS AND DISCUSSION

Flavodoxins are distinguished from other flavoproteins by their low redox potential and low molecular weight of approx. 15 000. They are synthesized well only under iron deficiency, contain FMN and can replace ferredoxin in photosynthetic NADP reduction of broken chloroplasts (1). Therefore this assay system was used to identify flavodoxin in our preparations, also obtained from low-iron cultures. Fig. 1 shows that flavodoxin from Chlorella in equimolar amounts is 60% as active as spinach or Chlorella ferredoxin. The replacement of ferredoxin by flavodoxin in vitro is amply matched by the occurence of both in vivo. Our results show that in cells (1 kg wet weight) from a complete culture medium (6) flavodoxin occurs beside ferredoxin in a molar ratio of 1:10. On the other hand under

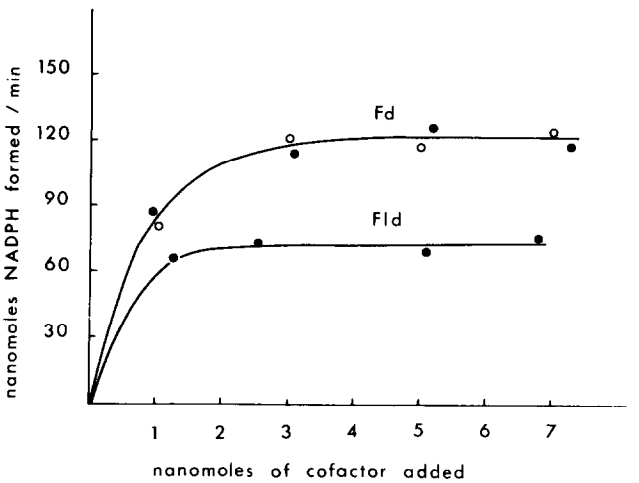

Fig. 1 Flavodoxin (Fld)- or ferredoxin (Fd)-catalyzed NADP reduction by spinach chloroplasts. A 1 cm cuvette contained in a volume of 2 ml 0.6 micromoles NADP, 100 micromoles Tris (pH 7.5), 20 micromoles $MgCl_2$, 0.1 mg chlorophyll and cofactors as indicated. Reference cuvette without cofactor; light intensity 30 000 lux. Upper curve ferredoxin from spinach or Chlorella, lower curve flavodoxin from Chlorella.

severely iron-deficient conditions no or only trace amounts of ferredoxin were detected, whereas appreciable quantities of cytochromes and the iron protein nitrite reductase (10,15) still were found. This points to a certain order of preference according to vital importance of iron proteins.

Fig. 2a shows the absorption spectrum of oxidized flavoprotein with absorption maxima at 275, 379 and 464 nm, respectively. Small shoulders were found at 292 and 490 nm. Characteristic absorption ratios are as follows, $A_{275\ nm}/A_{379\ nm}$ = 6.04 and $A_{275\ nm}/A_{464\ nm}$ = 5.46. The millimolar extinction coefficient at 464 nm is 10 ± 0.2. The spectrum is typical for protein-bound flavin with a remarkable shift of the main flavin peak over approx. 20 nm. Anacystis phytoflavin exhibits the same absorption maxima (2,4) whereas the red absorption band of bacterial flavodoxins has a small blue shift (13,14). Under nitrogen atmosphere in the presence of 50 mM EDTA and Na-acetate (pH 5.6), and on irradiation with a tungsten actinic light source flavodoxin is slowly but reversibly reduced to the grey-blue semiquinone form (Fig. 2b). On raising the pH to 8 and further addition of formamidine sulfinic acid in 200-fold excess one can gradually observe the decrease of the semiquinone absorption and the appearance of the fully reduced form (Fig. 2c). Semiquinone maxima

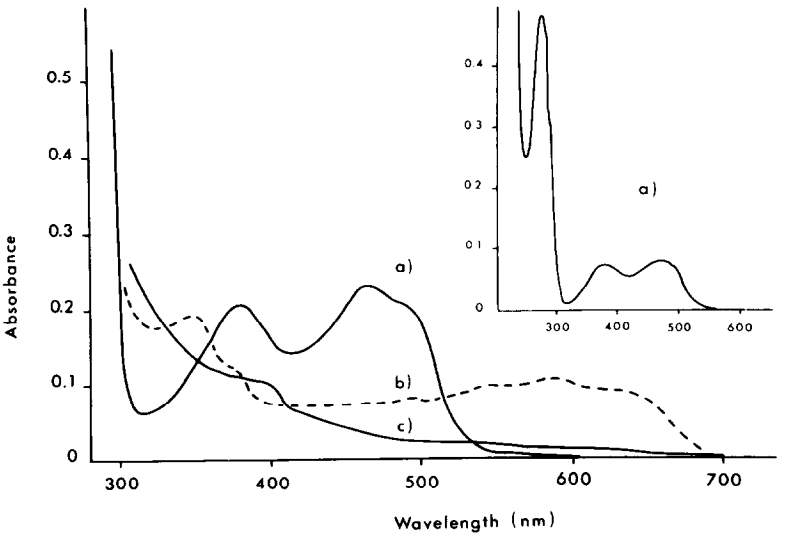

Fig. 2 Absorption spectrum of flavodoxin from Chlorella. Curve a) oxidized form, b) semiquinone form, c) reduced form; for details see the text.

are at 350 and 588 nm. Isosbestic points of the couple oxidized flavodoxin/semiquinone are at 359 and 517 nm. Isosbestic points of the couple fully-reduced flavodoxin/semiquinone are at 329 and 385 nm. Furthermore the fully reduced form shows a maximum at 395 nm. Readmittance of oxygen reverses the reduction. These observations are similar to those made by Massey and Palmer (16) and Mayhew and Massey with bacterial flavodoxin (14).

By using the electrophoretic technique in polyacrylamide with trypsin inhibitor (soybean), myoglobin, hemoglobin, ribonuclease and chymotrypsin we determined a molecular weight of 22 000 (see Fig.3). Besides, we found it rather surprising to get with this method even in most thoroughly purified preparations another band of just half the molecular weight. Experiments are in progress to reveal if this is due to a splitting of the molecule under those strongly dissociating conditions or an unusual behavior of Chlorella flavodoxin during the dodecylsulfate electrophoresis.

By gel filtration studies in Sephadex G-75 and using myoglobin, bovine serum albumin, chymotroypsinogen A and cytochrome c as standards we estimated an average molecular weight of 21 000. Amino acid composition indicated a molecular weight of

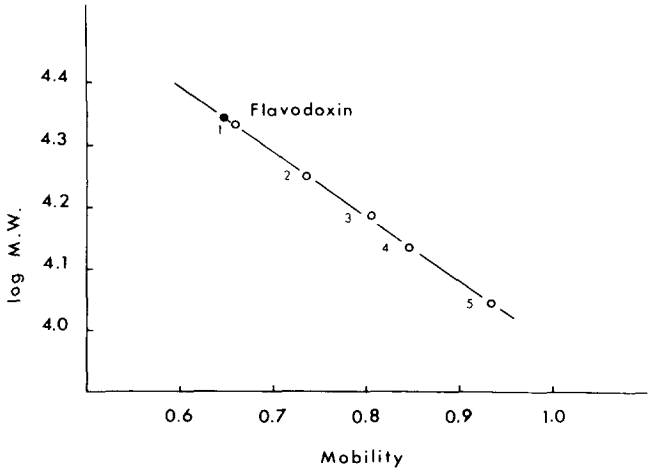

Fig. 3 Molecular weight determination of flavodoxin by dodecylsulfate electrophoresis. In each case 20 micrograms of protein were applied to the gel tube. Protein standards (1) trypsin inhibitor (soybean), (2) myoglobin, (3) hemoglobin, (4) ribonuclease and (5) chymotrypsin (11 000 chain plotted) were obtained from Serva (Heidelberg).

21 700. From these data we conclude a final molecular weight of 22 000 daltons.

The identification of the prosthetic group by paper chromatography disclosed FMN and not FAD as part of the protein. This is in agreement with findings on Anacystis phytoflavin (2) and bacterial flavodoxins (1). The quantitative estimation revealed a content of 1.1 µg FMN per 63 µg of protein. This corresponds to 1 FMN per 27 000 g of protein and suggests the binding of one FMN molecule per protein molecule.

The amino acid composition is shown in table I. According to the higher molecular weight in comparison with bacterial flavodoxins the molecule consists of 206 amino acids with a prevalence of certain amino acids such as aspartic and glutamic acid glycine and alanine. As regards comparable amino acids there is a good resemblance with those of bacterial flavodoxins.

In conclusion, the Chlorella flavoprotein seems to be a new member in the class of flavodoxins by its absorption spectrum, formation of the blue radical upon reduction, similarity of the

TABLE I

AMINO ACID COMPOSITION OF FLAVODOXINS

	Chlorella fusca[a)]	Clostridium pasteurianum (13)
	(residues per molecule)	
Lys	10	10
His	4	0
Arg	4	2
Asp	27	18
Thr	12	4
Ser	12	14
Glu	24	19
Pro	5	4
Gly	25	17
Ala	24	14
Half-cystine	5	1
Val	13	15
Met	1	4
Ile	8	5
Leu	18	12
Tyr	6	2
Phe	4	3
Trp[b)]	4	4
Total	206	148

[a)] calculated values from 20 and 70 h hydrolysis,
[b)] determined by hydrolysis with 2% thioglycolic acid (17).

amino acid composition and identity of the prosthetic group. It is only different in its slightly higher molecular weight. Further physico-chemical properties of the flavodoxin from Chlorella and some aspects of its physiological role in nitrate assimilation will be presented in a subsequent paper.

ACKNOWLEDGEMENTS

We wish to thank Prof. E. Kessler for his support during this work. Mrs. Manuela Chaves joined parts of this research. For reliable assistance we feel greately indebted. We thank also Dr. L. Gürtler for the amino acid analysis. Mr. H. Wurzbacher from the botanic garden kindly supplied us with spinach. We acknowledge the help of the Deutsche Forschungsgemeinschaft and the Stiftung Volkswagenwerk.

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