Vol. 81, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1978 Pages 630-635

A CONSTITUTIVE FLAVODOXIN FROM A EUKARYOTIC ALGA

Michael P. Fitzgerald, Ajimilah Husain and Lyndon J. Rogers

Department of Biochemistry, University College of Wales, Aberystwyth, U.K.

Received February 17,1978

SUMMARY:

We report the isolation and some properties of a flavodoxin from a eukaryotic organism, the naturally occurring red alga Chondrus crispus. Unlike the situation with most other organisms the flavodoxin, under normal growth conditions, is the predominantly formed low-potential electron carrier, an accompanying ferredoxin occurring in only very small amounts. The flavodoxin is of molecular weight 21000 and one mole of FMN is present per mole of protein. Reduction of the flavoprotein proceeds via a blue flavosemiquinone radical. The flavodoxin is active both in photosynthetic NADP reduction by broken chloroplasts, and in phosphoroclastic cleavage of pyruvate by cell-free extracts of Clostridium pasteurianum.

Flavodoxins are low molecular weight electron transfer proteins which have been shown to substitute for ferredoxin in a number of biological reactions (1,2). The first such flavoprotein was isolated from the cyanobacterium Anacystis nidulans (3) and its physiological role as a low potential electron transfer agent indicated by its ability to replace ferredoxin in light-dependent NADP reduction by higher plant chloroplasts (4). It has since been shown to substitute for ferredoxin in a number of other reactions including the phosphoroclastic oxidation of pyruvate (5).

Although flavodoxin is apparently a normal constituent of cells of Escherischia coli (6) and Azotobacter vinelandii (7) its production by other organisms, e.g. Clostridium pasteurianum (5) and the cyanobacteria Anacystis nidulans (4) and Nostoc strain Mac (8), is only induced by iron deficient conditions.

Zumft and Spiller (9) demonstrated that flavodoxins were not restricted to prokaryotic organisms, showing that flavodoxin formation in the green alga

0006-291X/78/0812-0630$01.00/0

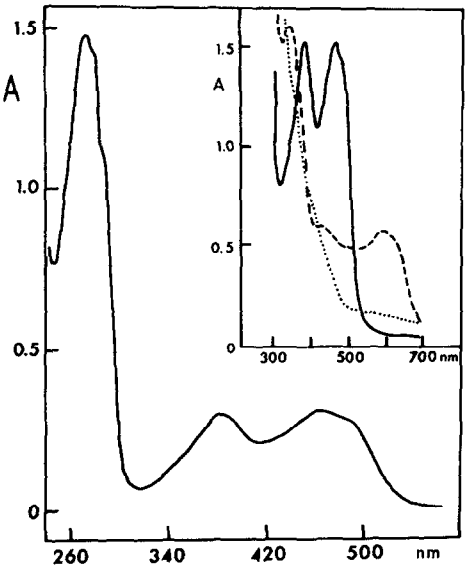

Fig. 1. Absorption spectrum of Chondrus crispus flavodoxin. The insertion shows the changes in spectrum on reduction of 420 nmoles flavodoxin in 300 mM Tris-HCl, 550 mM NaCl (pH 7.4) with sodium dithionite (840 nmoles). Reactions, in a total volume of 3.0 ml, were carried out under argon, and 1 μM methyl viologen was present as redox mediator. (——, fully oxidised; - - -, semiquinone; ..... fully reduced).

Chlorella fusca could be induced by iron deficient growth conditions. We have now found that the eukaryotic red seaweed Chondrus crispus (L.) Stakh possesses a constitutive flavodoxin.

Methods: Chondrus crispus was collected from local beaches (see ref. 10 for some sites and harvesting technique), and thoroughly washed. The fronds were then dried in a commercial freeze-drier before being ground to a fine powder using a laboratory mill. The powder was stored under dry conditions at -20°C until use. To isolate the flavodoxin the powder was suspended in 0.15 M Tris-HCl buffer at pH 7.4 (100g powder/l buffer), and stirred overnight. All operations were carried out at 5°C. Following brief centrifugation to remove solid debris DEAE-cellulose (30g/100g powder) was added to bind the flavodoxin present. The DEAE-cellulose was then packed into a column and the flavodoxin eluted by 0.3 M Tris-HCl containing 0.55 M NaCl, pH 7.4. Separation from small amounts of an accompanying reddish-brown ferredoxin and other protein was achieved by passage through a DEAE-cellulose column (3 x 30 cm) with elution by 0.15 M Tris-HCl containing 0.15 M NaCl, after washing with 0.15 M Tris-HCl. A final gel filtration on Sephadex G 100 yielded the yellow flavodoxin, which gave a single protein band following electrophoresis on 10%, 15% and 20% polyacrylamide gels. The final yield of flavodoxin was 20-30 mg from 600g freeze-dried powder. The flavodoxin was stable at -20°C for several weeks; however, prolonged storage in the absence of reducing agents resulted in some aggregation.

TABLE 1: PROPERTIES OF CHONDRUS CRISPUS FLAVODOXIN

Some results are given as Means ± S.D. for n experiments

Absorption maxima	275,(283),(292),386,464,(487)
Ratio of absorbance	1.0 0.21,0.21
Molar extinction coefficient at 464 nm ($M^{-1}cm^{-1}$)	10700
Prosthetic group	1 FMN
Sedimentation coefficient ($s_{20',w}$)	$2.15\pm0.1\times10^{-13}$ sec;n=5
Diffusion coefficient ($D_{20',w}$)	$9.1\pm0.25\times10^{-7}$ cm^2sec^{-1};n=3
Molecular weight	
by Svedberg equation	21300
by Sedimentation equilibrium	20800±1400;n=6
by Amino acid composition	22500
Partial specific volume	0.73
Isoelectric point	3.5±0.1;n=4
Biological activity	
NADP photoreduction (cf. pea ferredoxin)	0.65
Phosphoroclastic reaction (cf. Clostridium ferredoxin)	0.47

FMN was identified and estimated after dissociation from the apoprotein by trichloroacetic acid (11).

Other methods are as described elsewhere (12).

RESULTS AND DISCUSSION

The pure flavodoxin is bright yellow with absorbance peaks at 386 nm and 464 nm, and a shoulder at 487 nm. The major peak in the ultra-violet is at 275 nm, with pronounced shoulders at 283 nm and 292 nm. The 464 nm/275 nm absorbance ratio, used as a convenient criterion of purity, was typically 0.21 and occasionally somewhat higher.

The flavodoxin could be reduced to the blue semiquinone form by prolonged illumination in the presence of high concentrations of EDTA (13) or by addition of one equivalent of dithionite; a further equivalent of dithionite gave the hydroquinone (Fig. 1). Even in the presence of methyl viologen as mediator the reduction by dithionite was slow.

The prosthetic group, released from the protein by boiling for 10 min or by 5% w/v trichloroacetic acid, in the dark (11), was identified as FMN by co-chromatography with authentic standards in three solvent systems using both paper and thin-layer chromatography (5). The purified protein contains no iron or acid-labile sulphide.

From measurements of the FMN dissociated from a known weight of flavodoxin, and the calculated molecular weight of 21000, there is one FMN bound per molecule of protein. This had been suggested by the molar extinction coefficient of the flavodoxin at 464 nm (Table 1). The absorption of the main peak of the free flavin is shifted 20 nm toward longer wavelengths and in this respect the flavodoxin behaves similarly to that from the green alga Chlorella fusca (9); in contrast, most bacterial flavodoxins show 2-3 nm shifts towards shorter wavelengths when the flavin is dissociated.

The flavodoxin sedimented in the analytical ultracentrifuge as a single symmetrical peak of sedimentation coefficient 2.15×10^{-13} sec. Observations of the diffusion of a boundary between protein and buffer formed in the ultracentrifuge using a synthetic boundary cell enabled diffusion coefficient to be calculated by the area-maximum height method. Substitution of this value ($9.1 \times 10^{-7} cm^2 sec^{-1}$) with that of sedimentation coefficient in the Svedberg equation gave a molecular weight of 21300. This was in good agreement with the value determined directly by meniscus depletion and low-speed equilibrium sedimentation. Neither sedimentation nor diffusion coefficients showed concentration dependence over the range 2-6 mg protein/ml. The molecular weight and some other physical properties are summarised in Table 1. Chondrus flavodoxin may be placed with Chlorella and Nostoc flavodoxins which have similar molecular weights, in contrast to the flavoproteins from C. pasteurianum and E. coli which have lower molecular weights of about 15000. The molecular weight of Anacystis nidulans flavodoxin is in doubt (7 cf. 14).

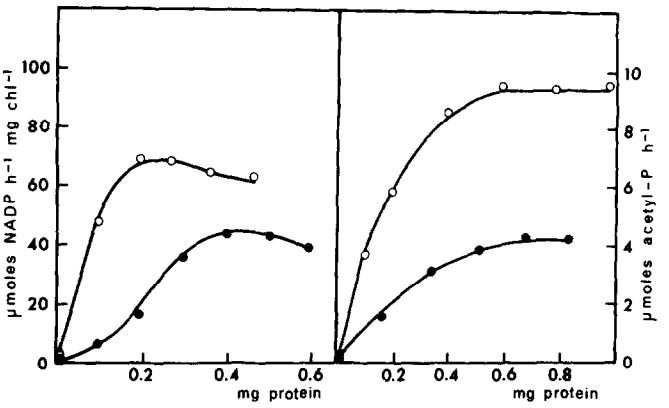

Fig. 2. Activities of Chondrus crispus flavodoxin. (a) NADP photoreduction by pea (Pisum sativum) chloroplasts illuminated with saturating light was followed spectrophotometrically. Chloroplasts were prepared by the method of Walker (15). The reaction mixture (3.0 ml) contained (in µmole): sucrose, 1000; NADP, 0.2; $MgCl_2$, 3; NH_4Cl, 3; Tris-HCl (pH 7.6), 150; the indicated amounts of pea ferredoxin (○), or Chondrus crispus flavodoxin (●), and chloroplasts equivalent to 30 µg chlorophyll; (b) phosphoroclastic reaction by Clostridium extracts. The phosphoroclastic oxidation of pyruvate by a ferredoxin-depleted cell-free extract of C. pasteurianum was followed by the formation of acetyl-phosphate. The reaction mixture (1.0 ml) contained (in µmole): NaH_2PO_4 (pH 6.5), 50; sodium pyruvate, 10; CoA, 0.13; the indicated amounts of C. pasteurianum ferredoxin (○) or Chondrus crispus flavodoxin (●), and cell extract equivalent to 5 mg protein. The reaction was stopped by addition of alkaline hydroxylamine.

The amino acid composition of the Chondrus flavodoxin is Lys_{11}; His_2; Arg_3; Try_6; Asx_{28}; Thr_{12}; Ser_9; Glx_{16}; Pro_{11}; Gly_{17}; Ala_{14}; Cys_1; Val_{14}; Met_3; Ile_8; Leu_{13}; Tyr_5; Phe_{11}. Chlorella flavodoxin has a markedly different amino acid composition (9) possessing, in particular, appreciably more alanine, cysteine and leucine, but much less proline, methionine and phenylalanine.

Chondrus flavodoxin is able to catalyse NADP photoreduction by isolated pea chloroplasts depleted of native ferredoxin and is also active in the phosphoroclastic oxidation of pyruvate catalysed by a ferredoxin-depleted C. pasteurianum extract (Fig. 2). In both cases the activity is comparable with that of flavodoxin from the cyanobacterium Nostoc strain Mac (G.N. Hutber, A.J. Smith and L.J. Rogers, unpublished).

Further studies on the physiological importance of the flavodoxin and its occurrence in other red seaweeds are in progress.

ACKNOWLEDGEMENTS: We thank the U.K. Science Research Council for financial support (Grant GR/A 1427-8). Miss M.M. Williams provided skilled technical assistance, and J.E. Dutton collaborated in some aspects of the work.

REFERENCES

(1) Yoch, D.C., and Valentine, R.C. (1972) Ann. Rev. Microbiol., 26, 139-162.

(2) Mayhew, S.G., and Ludwig, M.L. (1975) in P.D. Boyer, ed., The Enzymes, 12b, pp. 57-118, Academic Press, New York.

(3) Smillie, R.M. (1963) Plant Physiol. Suppl., 38, 28.

(4) Smillie, R.M. (1965) Plant Physiol., 40, 1124-1128.

(5) Knight, E., and Hardy, R.W.F. (1966) J. Biol. Chem., 241, 2752-2756.

(6) Vetter, H., and Knappe, J. (1971) Hoppe-Seyler's Z. Physiol. Chem., 352, 433-466.

(7) Van Lin, B., and Bothe, H. (1972) Arch. Mikrobiol., 82, 155-172.

(8) Hutber, G.N., Hutson, K.G. and Rogers, L.J. (1977) FEMS Microbiol. Lett., 1, 193-196.

(9) Zumft, W.G., and Spiller, H. (1971) Biochem. Biophys. Res. Commun., 45, 112-118.

(10) Marshall, S.M., Newton, L., and Parr, A.P. (1949) in A Study of Certain British Seaweeds and Their Utilization in the Preparation of Agar, HMSO, London.

(11) Mayhew, S.G., and Massey, V. (1969) J. Biol. Chem., 244, 794-802.

(12) Hutson, K.G., Rogers, L.J., Haslett., B.G., Boulter, D., and Cammack, R. (1978) Biochem. J. in press.

(13) Massey, V., and Palmer, G. (1966) Biochemistry, 5, 3181-3189.

(14) Smillie, R.M., and Entsch, B. (1971) Meth. Enzymol., 23a, 504-514.

(15) Walker, D.A. (1971) Meth. Enzymol., 23a, 211-220.