THE ISOLATION AND CHARACTERIZATION OF *RHODOSPIRILLUM RUBRUM* FLAVODOXIN

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Summary

The isolation and characterization of a flavodoxin from Rhodospirillum rubrum is reported. Chemical and physical measurements indicate a molecular weight of 23,000 g-mole^{-1}, one FMN# per mole of protein, and an amino acid composition similar, in many respects, to those of flavodoxins isolated from other organisms. R. rubrum flavodoxin is about 20% as active as spinach ferredoxin in mediating the photoreduction of $NADP^+$ by spinach chloroplasts.

INTRODUCTION

Flavodoxins have been isolated from Clostridium pasteurianum (1), Peptostreptococcus elsdenii (2), Desulfovibrio gigas (3), and Desulfovibrio vulgaris (4) when these organisms are grown in iron-deficient media. A low-molecular weight flavoprotein with ferredoxin activity, named phytoflavin, has also been isolated from the blue-green alga Anacystis midulans (5).

Ferredoxin has been detected in extracts of the facultative photoheterotroph Rhodospirillum rubrum (6) but has not been sufficiently purified for chemical and physical characterization. We report here the isolation and some of the chemical and physical properties of a flavodoxin produced by cells of R. rubrum which have been grown in an iron-deficient medium.

METHODS

Rhodospirillum rubrum (strain 2.1.1, Van Niel) was grown on a medium

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#Abbreviations: FMN - flavin mononucleotide
CD - circular dichroism

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consisting of the following reagents (per liter): succinic acid -4g, 2-(hydroxymethyl) - 1,3-propandiol -2.4g, K_2HPO_4-1g, $(NH_4)_2SO_4$-1g, $MgCl_2$-0.05g, casamino acids (Difco) - 1g, biotin - 7µg, and Larsen's trace elements without iron -1ml (7). The pH of the medium was adjusted to 7.4 before sterilization. Iron analysis of the medium (8) indicated a concentration of 350µg per liter. The organism was grown, using a 20% inoculum, in an illuminated water bath at 35° for 48 hr. The cells were then harvested and stored at -10° until used.

Absorption spectra were measured on a Cary Model 14R spectrophotometer. A Cary Model 60 spectropolarimeter equipped with the Model 6001 circular dichroism attachment was used in measuring CD spectra. Amino acid compositions were determined with a Beckman 121c amino acid analyzer following hydrolysis in 6N HCℓ (constant boiling) at 110° in sealed, evacuated tubes for various periods of time. The molecular weight was determined by the short column sedimentation equilibrium method of Yphantis (9) on a Beckman Model E analytical ultracentrifuge equipped with schlieren optics. The data were analyzed by a modification of the method of Van Holde and Baldwin (10).

The nature of the flavin moiety and the extinction coefficient of protein-bound flavin were determined by trichloroacetic acid treatment as described by Mayhew and Massey (2). The flavin semiquinone of R. rubrum flavodoxin was prepared photochemically according to the procedure of Massey and Palmer (11). Activities in the mediation of photoreduction of $NADP^+$ by spinach chloroplasts were carried out as described by Crawford and Jensen (12).

RESULTS

Isolation of Flavodoxin

Intact R. rubrum cells were suspended in 2 volumes of 0.1M Tris, pH 7.3. Cells were broken by passing the suspension through a Ribi Cell Fractionator (Ivan Sorvall, Norwalk, Connecticut) at 20,000 psi. Most of the bacteriochlorophyll was removed by centrifugation at 30,000xg for 30 min. The pellet

was washed with an equal volume of 0.1M Tris, pH 7.3 and the supernatants combined. The slightly turbid, orange supernatant was then centrifuged at 100,000xg for 1 hr. The resulting orange supernatant was absorbed on a Type 40 DEAE column (4 cm x 10cm) (Brown and Co.), which had been previously equilibrated with 0.1M Tris, pH 7.3. After washing with the equilibrating buffer, the flavodoxin band was stripped from the column with 0.5M NaCl-0.02M Tris, pH 7.3. The flavodoxin fraction was desalted by passage through a Sephadex G-25 column and applied to a Type 20 DEAE column (4cm x 10cm) equilibrated with 0.01M Tris, pH 7.3. The column was washed with 2 volumes of equilibration buffer and then 40 volumes of 0.1M Tris, pH 7.3. Flavodoxin was eluted with 0.2M Tris, pH 7.3, concentrated by pressure dialysis and chromatographed on a Sephadex G-100 (4 x 80cm) column with 0.02M Tris -0.5M NaCl, pH 7.3 as the eluting buffer. The flavodoxin eluted as a strongly retarded symmetrical peak with a 280nm/375nm spectral ratio of 4.8. The purified material, after concentration, was homogeneous by the criterion of cellulose acetate electrophoresis (pH 8.6, barbital

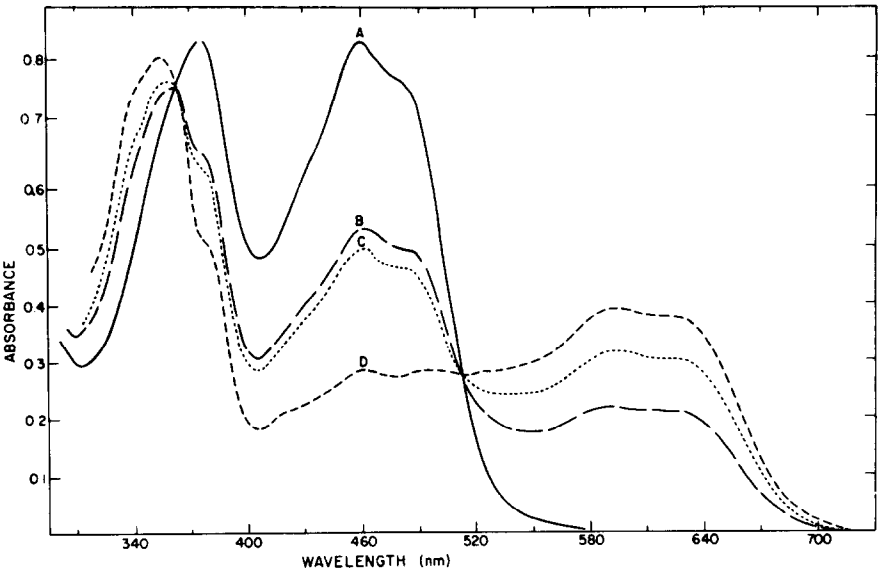

Figure 1. Absorption spectra R. rubrum flavodoxin. Buffer, 0.025 potassium phosphate - 0.05 M EDTA, pH 7.0. A (——) oxidized; B (- - -) partially reduced, following 35 hr of illumination; C (····) partially reduced, following 69 hr of illumination; D (----) semiquinone, formed by 120 hr of illumination, identical with species formed by reduction with an excess of sodium dithionite.

buffer). A yield of approximately 30mg of flavodoxin was obtained from 240g (wet weight) of cells.

Spectral Properties

The absorption spectra of R. rubrum flavodoxin during the course of photoreduction with EDTA is given in Figure I. Identical semiquinone absorption spectra were obtained whether produced photochemically or by dithionite reduction. The absorption maxima and corresponding extinction coefficients for the oxidized and semiquinone forms are given in Table 1. A neutral (blue) flavin semiquinone (11) is indicated by the absorption maxima at 627nm and at 588 nm. Apparently little or no flavin hydroquinone is formed during photoreduction as indicated from the isosbestic points at 513nm and at 363nm. The long wavelength absorption maximum of oxidized R. rubrum flavodoxin is red-

TABLE I

Extinction Coefficients and Absorption Maxima of R. rubrum Flavodoxin

Wavelength (nm)	Extinction Coefficient (M^{-1} cm^{-1})	
	Oxidized	Semiquinone
627		5,000
588		4,500
485 (shl)	10,300	
460	11,200	
376	11,300	
353		10,900
273		60,700
272	54,200	

shifted some 10-15nm when compared to the absorption maximum of P. elsdenii (2) and C. pasteurianum (1) flavodoxins; however, it is quite similar to the spectral maxima of those flavodoxins isolated from D. vulgaris and D. gigas (4).

Published CD data analyses in the visible region for a number of flavoenzymes (13) indicate similar CD spectra for the flavodoxins from P. elsdenii, Clostridium, R. rubrum and for the Shethna flavoprotein from Azotobacter vinelandii. The CD spectra in the visible and near ultraviolet regions of the oxidized and semiquinone forms of R. rubrum flavodoxin are given in Figure 2. The spectra are very similar to those of the Shethna flavoprotein both in shape

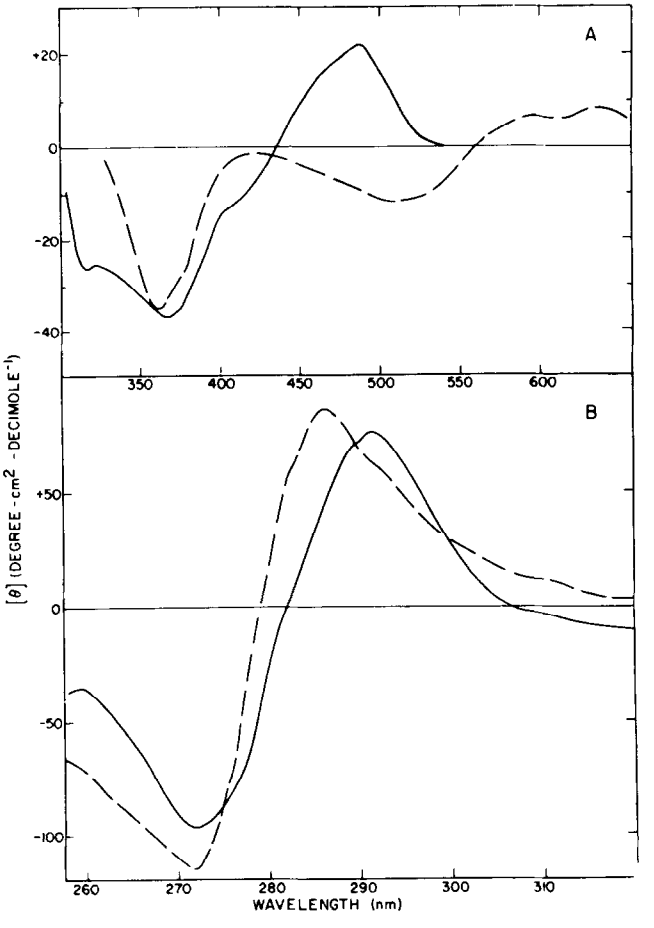

Figure 2. Circular dicroism spectra of R. rubrum flavodoxin. Buffer 0.025 M potassium phosphate - 0.05 M EDTA, pH 7.0. A. Visible region (——) oxidized, (- - -) semiquinone. B. Near UV, (——) oxidized, (- - -) semiquinone.

and intensity (13). Curve analysis of the oxidized form in the visible region (13) indicates R. rubrum flavodoxin to be more similar to the Shethna flavoprotein than to the P. elsdenii and Clostridial flavodoxins.

Redox Properties

Besides the spectral similarities, R. rubrum flavodoxin also resembles the Shethna flavoprotein (14,15) in that the flavin group can be reduced only to the semiquinone form upon addition of a large excess of dithionite at neutral pH. This may result from the ionization behavior of the flavin hydroquinone as suggested for the Shethna flavoprotein (14). The slow rate of photoreduction to semiquinone and of semiquinone oxidation by oxygen (14) also emphasizes the similarities between the two flavoproteins.

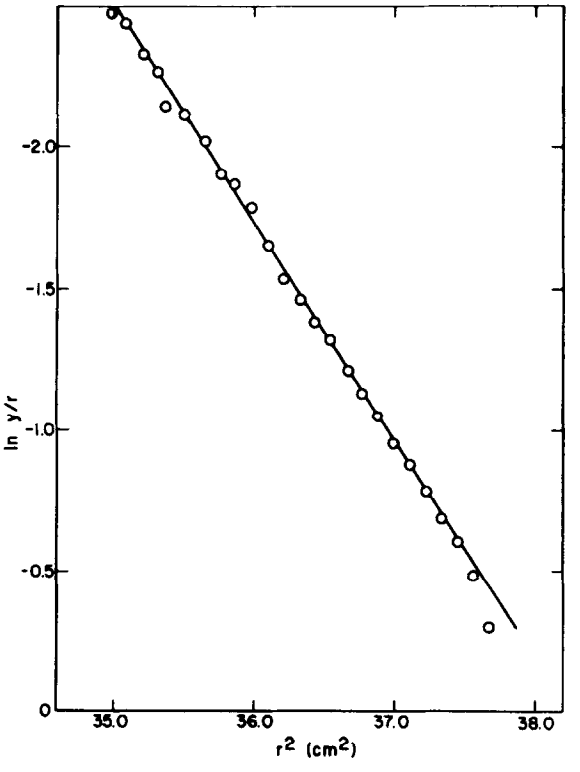

Figure 3. Sedimentation equilibrium centrifugation of R. rubrum flavodoxin. Plotted is log y/r vs. r^2. Buffer - 0.05 M potassium phosphate, pH 7.0, 20°C, 20,000 rpm, protein concentration 2 mg/ml. The data plotted was obtained after 27 hr of centrifugation.

Molecular Weight

The molecular weight of R. rubrum flavodoxin, as determined by sedimentation equilibrium, was found to be 22,800 ± 1,400 g-mole^{-1}. A $\bar{v}$ of 0.725 was used in the calculations. The linearity of the plot of ln y/r vs r^2 (Figure 3) indicates homogeneity. This flavodoxin is larger than those reported to date (M.W. = 15,000 g-mole^{-1}) but is similar in size to the Shethna flavoprotein (16)

TABLE II

Amino Acid Composition of

Rhodosprillum rubrum Flavodoxin

Amino Acid	Time of Hydrolysis (Hr)			Integral Value
	24	36	48	
Asp	24.8	24.8	23.0	25
Thr	15.4	15.1	15.4	16
Ser	10.3	10.1	10.3	11
Glu	15.9	15.9	16.0	16
Pro	4.8	6.9	6.4	7
Gly	27.6	27.6	26.0	28
Ala	19.8	20.5	19.6	20
Cys/2	1.4	1.2	0.8	2
Val	14.0	15.3	15.4	15
Met	2.0	2.2	2.0	2
Ileu	9.0	9.8	9.5	10
Leu	21.6	22.2	22.3	22
Tyr	9.0	9.0	9.0	9
Phen	5.5	5.7	5.3	6
His	1.4	1.3	1.1	1
Lys	9.0	9.0	9.0	9
Arg	6.9	7.4	7.6	8
Try				3*
Total				210

*Estimated from comparison of the absorption spectra with that of other flavodoxins.

Chemical Composition

The flavin can be dissociated from the protein moiety by treatment with 3% trichloracetic acid. Thin layer chromatography of the neutralized flavin solution (2) identified it as FMN.

Table II gives the amino acid composition of R. rubrum flavodoxin. This composition was calculated using the determined molecular weight of 23,000 $g\text{-}mole^{-1}$. In accord with the amino acid compositions reported for the other flavodoxins (2,4,17) and for the Shethna flavoprotein (16), there is a predominance of acidic and non-polar residues. D. vulgaris flavodoxin (4) is the only other flavodoxin reported that contains a histidine residue.

TABLE III

Flavodoxin Mediated $NADP^+$ Reduction

By Illuminated Spinach Chloroplasts

Sample	Activity	% Activity
	μM $NADP^+$ Reduced / mg Chlorophyll/hr	
Spinach ferredoxin	31.6	100
R. rubrum flavodoxin	5.8	18.3
P. elsdenii flavodoxin	12.6	40.0
Shethna flavoprotein	0	0

Biological Activity

The ability of R. rubrum flavodoxin to function in the place of ferredoxin was tested in the photoreduction of $NADP^+$ by isolated spinach chloroplasts. The results in Table III indicate R. rubrum flavodoxin was approximately 20% as effective as spinach ferredoxin in the assay. By comparison, P. elsdenii flavodoxin (a gift from Dr. S. G. Mayhew, Department of Biological Chemistry, The University of Michigan) was about 40% as effective while the Shethna flavoprotein, in agreement with previous results (15), indicated no activity.

DISCUSSION

The isolation of a flavodoxin from R. rubrum demonstrates a genetic adapt ability, in response to limiting iron, for the photosynthetic bacteria. Previously, only non-photosynthetic anaerobes were known to exhibit this behavior. The physical and chemical properties of this flavoprotein are similar to those of other flavodoxins, yet are more closely related to the Shethna flavoprotein which does not function as a flavodoxin. A detailed study of R. rubrum flavodoxin comparing it with the other flavoproteins would be of importance in determining the structural factors involved in flavodoxin biological activity.

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