EVIDENCE FOR A NOVEL FLAVIN PROSTHETIC GROUP ASSOCIATED WITH NADH DEHYDROGENASE FROM *PEPTOSTREPTOCOCCUS ELSDENII*

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SUMMARY

1. NADH dehydrogenase (EC 1.6.99.3) has been purified from the strictly anaerobic rumen bacterium *Peptostreptococcus elsdenii*. The purified protein is specific for NADH as electron donor; it can use 2,6-dichlorophenolindophenol (DCIP), cytochrome *c*, $K_3Fe(CN)_6$ or flavodoxin as electron acceptor. It has a molecular weight of about 63 000. The absorption spectrum shows maxima at 475, 375, 356, 318, and 273 nm. It has shoulders at 425, 460, and 510 nm. The protein is fluorescent with an emission maximum at 550 nm.

2. The chromophore is released from the NADH dehydrogenase by either heat treatment or extraction with trichloroacetic acid. The free chromophore shows an increase of absorption in the visible spectrum and a shift of the maximum to 470 nm. The fluorescence emission maximum is shifted to 530 nm, but the intensity is unchanged.

3. Hydrolysis of the free chromophore with phosphodiesterase causes a 12% increase in the visible absorption and a 2-fold increase in the fluorescence (exciting light at 480 nm). The chromatographic behavior is also affected by this treatment.

4. The hydrolysed chromophore is bound by apoflavodoxin with 95% quenching of the fluorescence and an 18% decrease in the visible absorption. The complex of apoflavodoxin and the unknown chromophore is reduced by light irradiation in the presence of EDTA to an intermediate similar to flavodoxin semiquinone but with a distinctively different absorption spectrum. It is concluded that the chromophore in the NADH dehydrogenase is a flavin dinucleotide similar to FAD but modified in the isoalloxazine ring.

INTRODUCTION

When crude extracts of the strictly anaerobic bacterium *Peptostreptococcus elsdenii* are treated on a column of DEAE-cellulose, a number of colored bands are separated as the ionic strength of the eluting buffer is increased. Four of these colored proteins, separated from extracts of cells grown in iron-poor medium[1], have

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

been identified. In order of their elution from the column, they are: NADH de-hydrogenase (EC 1.6.99.3); rubredoxin[2]; flavodoxin[1]; and butyryl-CoA dehydro-genase (EC 1.3.99.2) (P. C. ENGEL AND S. G. MAYHEW, unpublished observations). These proteins are colored yellow, red, yellow and green, respectively. The NADH dehydrogenase, and the chromophore associated with it, are the subjects of this communication.

An assay involving NADH oxidation and the reduction of 2,6-dichlorophenol-indophenol (DCIP) was used to follow activity during further purification of the NADH dehydrogenase to about 90% purity. The absorption spectrum of the purified material suggested the presence of flavin in the protein, but it was different in many respects from the absorption spectra of other simple flavoproteins. The experiments described in this paper suggest that the light absorption characteristics are due to a chromophore which is a flavin dinucleotide, but which is modified in the isoalloxazine ring. This evidence for such a modified flavin is of interest because to our knowledge only one other enzyme (succinate dehydrogenase, EC 1.3.99.1) has been shown to contain flavin modified in the isoalloxazine ring[3].

MATERIALS AND METHODS

Growth of P. elsdenii

P. elsdenii, strain LC 1[4], was maintained according to WALKER[5]. Cultures of 40 l were grown in iron-poor medium as described previously[1].

Enzyme assay

The assay used to measure diaphorase activity involved the oxidation of NADH and the reduction of DCIP. Assay mixtures contained in 3 ml; 0.05 M sodium phos-phate buffer (pH 7.3); $4 \cdot 10^{-5}$ M DCIP; and $1 \cdot 10^{-4}$ M NADH. The temperature was 25°. The reaction was initiated by addition of NADH dehydrogenase and was followed at 600 nm. One unit of NADH dehydrogenase is defined as the amount to cause an absorbance change of 1.0 per min at 600 nm.

Purification of NADH dehydrogenase

The early steps in the purification of NADH dehydrogenase were as described previously for the purification of flavodoxin from *P. elsdenii*[1]. Dried cells of the organism (50 g) were extracted in water, and the extract treated on a column of DEAE-cellulose (44 cm × 8 cm) prepared as described by MORTENSON *et al.*[6]. The column was eluted batchwise with 0.05 M Tris–HCl buffer (pH 7.3) containing NaCl. It was first eluted with 0.05 M Tris–HCl (pH 7.3) (4 l) and then with 0.05 M Tris–HCl (pH 7.3) containing successively 0.05 M NaCl (4 l), 0.1 M NaCl (4 l), 0.15 M NaCl (2 l) and 0.2 M NaCl. NADH dehydrogenase was collected as a yellow solution when the NaCl in the eluting buffer was 0.2 M. The NADH dehydrogenase solution was then fractionated with $(NH_4)_2SO_4$. Protein which precipitated between 35 and 70% saturated $(NH_4)_2SO_4$ was dissolved in about 20 ml 0.1 M potassium phosphate buffer (pH 6.8) and dialysed against 1 l of the same buffer. After dialysis this solution was divided into two parts and each part treated separately on a column of Sephadex G-100 (96 cm × 2.5 cm) equilibrated with 0.1 M potassium phosphate (pH 6.8). Fractions from these two columns which had the highest specific activity (23–32

units/$A_{275\ nm}$) were combined and fractionated with $(NH_4)_2SO_4$. The yellow protein which precipitated between 50 and 70% saturated $(NH_4)_2SO_4$ was dissolved in about 6 ml 0.1 M potassium phosphate (pH 6.8), the solution dialysed against the same buffer, and treated again on Sephadex G-100 as described above. Table I summarizes the steps in the purification procedure. Approx. 25% of the NADH dehydrogenase activity from the DEAE-cellulose column was recovered after the second Sephadex step. The yield of protein was 70.3 mg.

Thin-layer chromatography

Thin-layer chromatography of flavins was performed with either cellulose powder plates (MN-Polygram Cel 300) and 5% (w/v) Na_2HPO_4 (ref. 16), or with silica gel plates (MN-Polygram Sil S-HR from Brinkman Instruments Inc., Westbury, N.Y.) and n-butanol–acetic acid–water (4:3:3, by vol.)[16] as developing solvent. Flavins were detected by their fluorescence in ultraviolet light.

Determination of protein and AMP

Protein was determined by the biuret reaction[7]. AMP was measured enzymatically as described by KALKAR[8].

Materials

NADH was from P–L Biochemicals. Horse heart cytochrome *c*, alkaline phosphatase (calf mucosa, Type I) and adenosine deaminase (calf mucosa, Type II) were obtained from Sigma, chymotrypsinogen and ovalbumin from Worthington, and bovine plasma albumin from Armour Pharmaceutical Co., Kaukakee, Ill. For DEAE-cellulose chromatography, Whatman DE 32 was used. Flavodoxin and apo-flavodoxin were prepared from *P. elsdenii* as described previously[1].

RESULTS AND DISCUSSION

Properties of NADH dehydrogenase

NADH dehydrogenase prepared as described in MATERIALS AND METHODS is estimated to be 90% pure. It gives three protein bands after electrophoresis in acryl-amide gel at pH 9.2; a major yellow-colored band which is stained red by NADH and *p*-iodonitrotetrazolium violet, and two minor colorless bands which are inactive. The purified enzyme reduces DCIP, cytochrome *c* and ferricyanide, and it also catalyses a slow reduction of flavodoxin. It is specific for NADH; no reduction of these electron acceptors was observed with NADPH as substrate. The enzyme does not couple NADH oxidation with crotonyl-CoA reduction in the presence of butyryl-CoA dehydrogenase. Thus it is probably different from the Fraction II of BALDWIN AND MILLIGEN[9] which also shows NADH dehydrogenase activity. The molecular weight of the protein, determined with a calibrated column of Sephadex G-150[10] is about 63 000 (Fig. 1).

The absorption spectrum of the protein (Fig. 2) shows absorption maxima at 475, 375, 356, 318 and 273 nm. It also has marked shoulders at 425, 460 and 510 nm. The ratio of absorption at 273 nm to the absorption at 475 nm is 5.2. The visible absorption is decreased on anaerobic treatment with NADH, or by irradiation with visible light in the presence of EDTA (Fig. 3). There was no evidence for a semi-
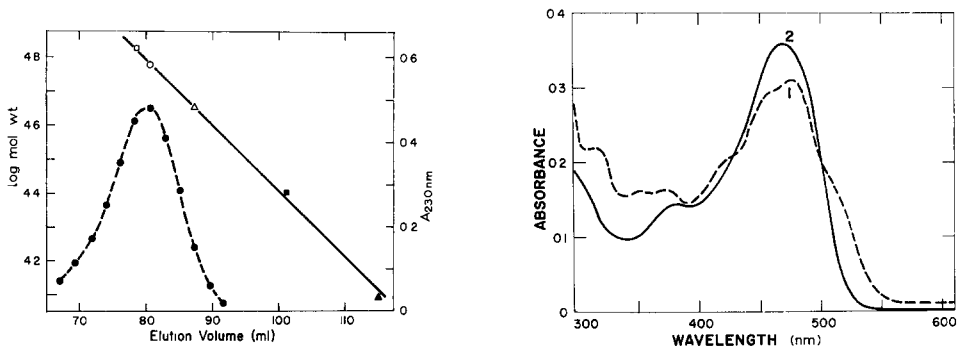
Fig. 1. Molecular weight of NADH dehydrogenase by gel filtration. — — —, absorbance at 230 nm of NADH dehydrogenase as a function of the elution volume, ———, log of the molecular weight as a function of the elution volume; □, bovine plasma albumin; ○, NADH dehydrogenase; △, ovalbumin; ■, chymotrypsinogen, ▲, horse heart cytochrome c. A Sephadex G-150 column (96 cm × 1.5 cm) was equilibrated at 4° with 0.1 M potassium phosphate (pH 6.8). The flow rate was about 5 ml/h.

Fig. 2. Absorption spectra of diaphorase and neutralized trichloroacetic acid extract from NADH dehydrogenase. NADH dehydrogenase, 0.54 ml, was mixed at 0° with 0.06 ml of 50% (w/v) tri-chloroacetic acid. After 5 min, the mixture was centrifuged (10 000 × g for 10 min) and the supernatant transferred to a 2-ml volumetric flask. The precipitate was resuspended in 0.6 ml of 5% trichloroacetic acid and the mixture centrifuged as before. The supernatant from this extraction was added to the first, and the white precipitate was discarded. The combined supernatant solutions were neutralized by adding 0.6 ml of M K_2HPO_4 and the volume was made up to 2 ml with water. Curve 1, untreated NADH dehydrogenase $(2.19 \cdot 10^{-5}$ M); Curve 2, trichloroacetic acid extract of NADH dehydrogenase $(2.19 \cdot 10^{-5}$ M).

quinone intermediate during reduction by these methods, a feature which distinguishes this protein from many other flavoproteins which show either red- or blue-colored semiquinones[11]. Aeration of the reduced NADH dehydrogenase caused a rapid reoxidation to the spectrum shown by Curve 1 in Fig. 3. The protein is quite strongly fluorescent with a fluorescence emission maximum at 550 nm and fluorescence excitation maxima at 480, 375, and 325 nm (Fig. 4). These absorption and fluorescence properties are not typical of simple flavoproteins which usually have absorption maxima at about 450 and 375 nm, and which in the rare cases where fluorescence is observed, have emission maxima at about 530 nm. However, there is strong presumptive evidence that the chromophoric group in this protein is in fact a flavin.

Properties of the free chromophore

The chromophore is released from the protein by either precipitation of the apoprotein with 5% trichloroacetic acid at 0°, followed by neutralization of the extract with K_2HPO_4, or by heat treatment in darkness in a sealed tube at 80° for 20 min. These treatments cause similar changes in the absorption and fluorescence properties of the chromophore (Figs. 2 and 4). The free chromophore shows an increase of absorption of the visible maximum and a shift of this maximum to 470 nm. The shoulders in the visible region disappear, and only a single absorption maximum remains at wavelengths shorter than 400 nm. This maximum is at 380 nm. The fluorescence emission maximum is shifted to 530 nm in the free chromophore, but
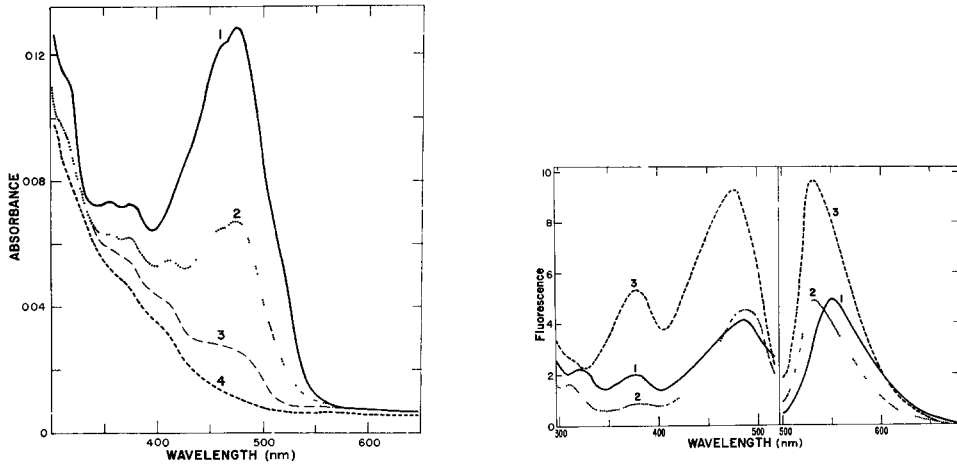
Fig. 3. Effect of light irradiation on NADH dehydrogenase in the presence of EDTA. A solution containing NADH dehydrogenase, 0.15 M EDTA and 0.12 M sodium phosphate (pH 7.8) was made anaerobic[11] in a stoppered 1-ml spectrophotometer cuvette. Absorption spectra were recorded after irradiating the cuvette with visible light for known time intervals at 4° (see ref. 1). Curve 1, before light irradiation; Curves 2–4, after 1.5, 5.5 and 316 min light irradiation, respectively.

Fig. 4. Fluorescence spectra of the chromophore. Curves on the left are excitation spectra (monitored at 535 nm) for (1) untreated NADH dehydrogenase, (2) NADH dehydrogenase denatured by heating for 20 min, at 80° in a sealed tube, and (3) denatured NADH dehydrogenase after addition of phosphodiesterase. Curves on the right are the emission spectra (excited at 480 nm) of the same samples. All three samples were equivalent to $5.65 \cdot 10^{-6}$ M NADH dehydrogenase. Fluorescence is shown in arbitrary units. Fluorescence measurements were made with a ratio recording fluorimeter[15] thermostated at 20°.

the fluorescence intensity is unchanged. The fluorescence excitation spectrum shows decreased absorption below 400 nm compared with the native protein.

Treatment of the chromophore with phosphodiesterase (*Naja naja* venom) causes a 12% increase in the absorption at 470 nm and marked changes in the fluorescence properties. The position of the fluorescence emission maximum does not change, but the intensity increases 2-fold when the exciting light is at 480 nm. The fluorescence excitation spectrum shows a 6.4-fold increase in intensity at 375 nm.

TABLE I

PURIFICATION OF NADH DEHYDROGENASE

Purification step	Vol. (ml)	Total activity (units)	Total $A_{275\,nm}$	Yield (%)	Specific activity (units/ $A_{275\,nm}$)
1. DEAE-cellulose eluate*	965	7900	1152	100	6.9
2. $(NH_4)_2SO_4$ fractionation	20.5	5850	631	74	9.3
3. 1st Sephadex column	83	4280	142	54	30.2
4. 2nd Sephadex column	32.5	2150	60.5	27	35.6

* The starting material was 50 g dry cells.

TABLE II

ANALYSES FOR AMP

Reaction mixtures contained in 0.82 ml, 0.1 M glycylglycine buffer (pH 8.4), the unknown chromophore (approx. $2 \cdot 10^{-5}$ M), 0.1 mg alkaline phosphatase and 0.1 mg adenosine deaminase. The temperature was 25°. The reaction was begun by addition of alkaline phosphatase and adenosine deaminase and followed to completion at 265 nm[8]. The change in absorption was measured *versus* a reagent blank from which the unknown chromophore was omitted.

	Moles of AMP per mole of chromophore
Heat supernatant before hydrolysis	0
Heat supernatant after hydrolysis	1.1–1.2

These changes suggest that, like the hydrolysis of FAD to FMN, the unknown chromophore is hydrolysed by phosphodiesterase from a dinucleotide to a mono-nucleotide; increases in absorption and fluorescence are also observed when FAD is hydrolysed to FMN[12]. Analysis of the products of hydrolysis for AMP[8], one of the products expected from the cleavage of an FAD-like compound, showed that one molecule of AMP was formed per molecule of chromophore hydrolysed (Table II). Thin-layer chromatography of the chromophore in two different solvent systems before and after hydrolysis with phosphodiesterase showed that material which gave a single fluorescent spot with an R_F similar to that of FAD was converted into a compound which gave a single spot with an R_F close to that of FMN (Table III).

Further evidence for such an hydrolysis was obtained from binding studies with apoflavodoxin. The apoprotein from *P. elsdenii* flavodoxin is very specific for FMN and FMN derivatives which it binds with almost complete quenching of the flavin fluorescence[13]. A complex between this apoprotein and FAD or riboflavin has not been observed. Apoflavodoxin does not bind the chromophore released directly from the NADH dehydrogenase. However, after treatment with phosphodiesterase the chromophore is bound very strongly. The fluorescence of the chromophore is

TABLE III

THIN-LAYER CHROMATOGRAPHY OF THE UNKNOWN CHROMOPHORE

Solvent system 1 was *n*-butanol–acetic acid–water (4:3:3, by vol.)[16]. Silica plates were used with this solvent system. For this experiment the unknown chromophore was a neutralised trichloro-acetic acid extract from the diaphorase. Solvent system 2 was 5% (w/v) Na_2HPO_4 (ref. 16). Cellulose plates were used with this solvent system. The unknown chromophore for this experi-ment was removed from the diaphorase by heat treatment as described in the text. The unknown chromophore was hydrolysed with phosphodiesterase.

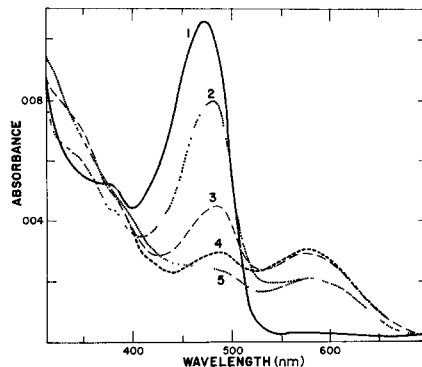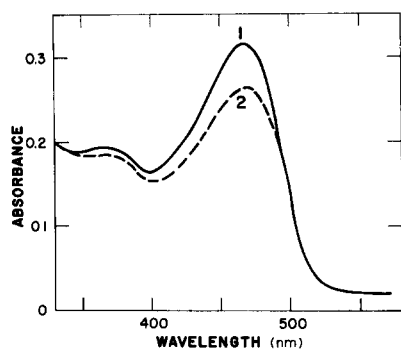|	R_F	
	Solvent 1	Solvent 2
FAD	0.41	0.43
FMN	0.54	0.54
Unknown chromophore	0.43	0.36
Unknown chromophore after hydrolysis	0.54	0.49

Fig. 5. Effect of apoflavodoxin on the absorption spectrum of the hydrolysed chromophore. Curve 1, 1.53·10⁻⁵ M chromophore hydrolysed with phosphodiesterase; Curve 2, 1.53·10⁻⁵ M hydrolysed chromophore *plus* 1.9·10⁻⁵ M apoflavodoxin. Samples were dissolved in 0.04 M potassium phosphate (pH 6.8).

Fig. 6. Light irradiation of the complex of apoflavodoxin and hydrolysed chromophore in the presence of EDTA. A solution of the complex of apoflavodoxin and the hydrolysed chromophore in 0.15 M EDTA and 0.015 M sodium phosphate (pH 7.3) was irradiated with visible light as described in the legend to Fig. 3. Curve 1, before light irradiation ,Curves 2–5, after 45, 109, 230 and 1030 min light irradiation, respectively.

quenched by 95% during binding, and the visible absorption maximum decreases by 18% (Fig. 5). Binding is evidently tight since extensive dialysis *versus* 0.03 M phosphate buffer (pH 7.3) causes no changes in the visible absorption spectrum of the complex. From spectrophotometric and fluorometric titration experiments with apoflavodoxin of known concentration, and by assuming that only one molecule of the chromophore is bound per molecule of protein, the concentration and extinction coefficients of the chromophore were determined. The extinction coefficient for the chromophore bound to the NADH dehydrogenase is 14 100 $M^{-1}\cdot cm^{-1}$ at 475 nm, 17 300 $M^{-1}\cdot cm^{-1}$ at 470 nm when released from the protein, 19 600 $M^{-1}\cdot cm^{-1}$ after hydrolysis with phosphodiesterase and 16 100 $M^{-1}\cdot cm^{-1}$ at 470 nm on binding the mononucleotide to apoflavodoxin.

Like native flavodoxin[1], and the complex of apoflavodoxin with iso-FMN[13], the complex of apoflavodoxin and the unknown chromophore can be reduced by irradiation with visible light in the presence of EDTA[11]. An intermediate is generated during the reduction (Fig. 6). This intermediate, which is probably analogous to the semiquinone intermediate seen during the reduction of native flavodoxin, differs from the semiquinone of native flavodoxin in having only a single broad absorption maximum at 580 nm, with no evidence for a shoulder at 610 nm[1]. The extinction coefficient for the intermediate at 580 nm, determined by plotting the absorption at 470 nm *versus* the absorption at 580 nm after different periods of light, was found to be 3800 $M^{-1}\cdot cm^{-1}$. Aeration of the reduced complex caused a slow reoxidation and a return of the spectrum of the untreated material.

While there is good circumstantial evidence that this chromophore is a flavin, perhaps modified in the isoalloxazine ring, the nature of the modification is not known. However, it should be noted that the absorption spectrum of the native diaphorase resembles the spectrum of 2-thio-substituted flavins[14]. 2-Thio-substituted

flavins are known to be rather unstable, and if the chromophore is indeed a flavin modified at the 2 position, it is possible that the methods used to release the chromophore from the diaphorase cause some further modification. Chemical studies are being carried out in an attempt to identify this chromophore.

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