한국농화학회지 Vol. 16, No. 1 Mar. 1973
J. Korean Agricultural Chemical Society

Electron Transport Carrier for the Free Radical Shethna Flavoprotein in *Azotobacter vinelandii*

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(Received Jan. 20, 1973)

Azotobacter vinelandii Shethna Flavoprotein의 Free Radical 生成을 爲한 電子傳達物質

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SUMMARY

Azotobacter vinelandii cell extracts and its variety of purified fractions with regard to their ability to form the redox state of the Shethna Flavoprotein (free radical form FPH·) were studied. A fluorescent flavoprotein (protein I) and a brown protein (protein II) were the most active proteins which were isolated in purified form. The free radical formation activity was substantially decreased during the purification and was completely lost upon storage in a week under nitrogen in a frozenstate. The presence of free flavin (FMN) with NADH enhanced the rate of free radical formation. The reaction of FMN and NADH was found to be catalysed by various cell fractions.

A possible role of FMN as a substrate for free radical shethna flavoprotein was investigated.

Slower reaction rate of $FMNH_2$+Flavoprotein (FP) —→ FPH+FMN than FMN+NADH —→ $FMNH_2$, accumulation of $FMNH_2$ ocurred which subsquently caused FPH·

INTRODUCTION

One of the flavoproteins in *Azotobacter vinelandii*, known as the Shethna flavoprotein, has been the object of appreciable study because of the unusual stability of its half-reduced radical form towards oxygen.[1,2] The Shethna flavoprotein is present in a cell free extract of *Azotobacter vinelandii* as a mixture of half-reduced (blue) and oxidized forms (yellow). An ESR signal at $g=2.0$ can be observed both in whole cells and in a cell-free extract under aerobic conditions. Some of its physical and chemical properties are known; its molecular weight is 23,000, and its spectroscopic properties are well established.[2,3] On the other hand, the biological function of the Shethna flavoprotein is still uncertain, although recent work (Arnon) suggests a possible role as an electron

donor in the nitrogen fixation process. In order to obtain information concerning the natural electron donor from which the free radical form of the flavoprotein is generated, we have studied *Azotobacter* extracts and a variety of purified fractions with regard to their ability to form this redox state of the Shethna protein.

MATERIALS and METHODS

Reagents

NADH was obtained from Calbiochem; TPNH, Riboflavin, FMN, FAD and Trizma base were from Sigma Chemical Co. DEAE (fibrous) and Bio-gels were obtained from Calbiochem and DE-32(DEAE microgranular) from Whatman Paper Co. All other chemicals used throughout the experiments, of highest purity available, were obtained commercially.

Instrumentation

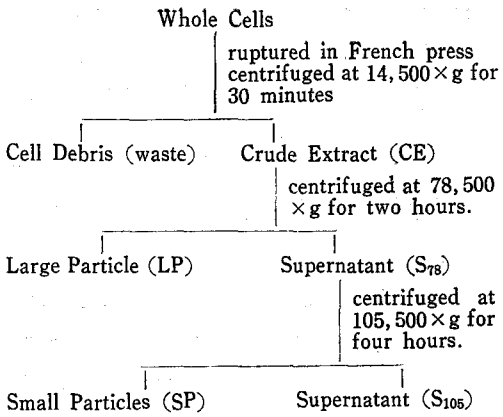
Absorbance measurements and absorption spectra were determined using the Gilford and Coleman-Hitachi 124 dual beam spectrophotometers. Centrifugations were carried out with a Sorval superspeed RC-2B type centrifuge and a Beckman Model L ultracentrifuge. Electron spin resonance measurements were obtained with a Varian V-4501 electron paramagnetic resonance spectrometer equipped with a Fieldial control.

Growth of *Azotobacter vinelandii*

Azotobacter vinelandii, strain OP, was grown in Burk's medium with aeration for 24 hours; the details are reported elsewhere.[1,4] The bacterial were harvested using a Sharples centrifuge and frozen until use.

Fractionation of *A. vinelandii* extract

The cells were suspended in 0.025M phosphate buffer (pH=7.4; the same buffer was used throughout these experiments unless otherwise specified), 1 : 3 by volume, and were broken in a French press (1,600 lb/in^2). The following fractions were separated by centrifugation: crude extract (CE), 14,500×*g*, 30 minutes; large particles (LP), 73,500×g, 2 hours and supernatant (S_{78}); small particles (SP) 105,500×g, 4 hours and supernatant (S_{105}). The overall flow diagram for the preparation is as follows:



For further fractionation, the S_{105} fraction was treated with ammonium sulfate and subjected to DEAE column chromatography. The S_{105} fraction was brought to 40% saturation of ammonium sulfate and the precipitate was sedimented by centrifugation at 14,500×g for 20 minutes, and washed with 40% saturated ammonium sulfate solution. The supernatant resulting from the 40% ammonium sulfate precipitation was brought to 60% saturation and the resulting precipitate was separated in the same way. The S_{105} fraction was thus separated into three fractions: 0-40%, 40-60 %, and 60% ammonium sulfate precipitates.

The ammonium sulfate precipitates were dialyzed against buffer and applied to a column of fibrous DEAE-cellulose equilibrated with 0.025M phosphate buffer. The column was eluted with stepwise increases in the concentration of the buffer system. Further purification was carried out on microgranular DEAE (DE-32, Whatman) column with gradient elution. DE-32 was precycled before using. The purity of the protein fractions was checked by polyacetate strip electrophoresis.

Preparation of Shethna flavoprotein

The Shethna flavoprotein (FP) was prepared by the method described by Hinkson and Bulen.[2]

Measurement of Flavoprotein Free Radical Formation

Measurement of flavoprotein free radical(FPH•) formation activity was done in a Thunberg cuvette, placing the flavoprotein in the main cell and the electron carrier protein in the side arm, or vice versa. The cuvette was evacuated and flushed

with N_2 gas. The reaction was started by mixing the solutions and the absorbancy change at 580 nm was read. Reactions were run at room temperature. When the reaction mixture consisted of more than tow components, cuvette with an additional side arm was used.

Measurement of NADH Oxidase

NADH oxidase activity was measured by the decrease in absorbancy at 340 nm with a Gilford spectrophotometer. The reaction was carried out in a 1cm cuvette which contained 0.2 m*l* of NADH solution (1.3×10^{-3}M), 0.1 m*l* of enzyme solution and 1.5 m*l* of 0.025 M phosphate buffer.

Measurement of NADH Dehydrogenase

The assay method was the same as for NADH oxidase, except that the reaction was done anaerobically in a Thunburg tube.

Measurement of Succinate Dehydrogenase

The assay procedure used was same as the method bescribed by Der Vartanian *et al.* [5], using ferricyanide as the electron acceptor.

Other Procedures

The protein content of samples was measured by the method of Lowry *et al.*[6] Concentration of enzyme solutions was done by using either a Diaflo ultrafiltration cell under N_2-pressure or Aquacide (Calbiochem Co.). Salts such as ammonium sulfate or sodium chloride were eliminated by either dialysis against phosphate buffer or by using Biogel P-10 or Sephadex 10 gel filtration.

RESULTS and DISCUSSION

Distribution of Flavoprotein Free Radical Formation Activity in Azotobacter vinelandii Cell Fractions

The absorption spectra of the various fractions prepared by centrifugation are shown in Fig. 1. The main absorbing materials appear to be non-heme iron proteins and cytochromes. The activities of the fractions in flavoprote in free radical formation are shown in Table 1. The S_{78} fraction was centrifuged at 105,500×g for four hours to give S_{105} and SP. The latter had most of the activity that the S_{78} fraction had, while S_{105} showed little activity. Fig. 2 shows some representative time courses of FPH· formation. Note that these are quite slow.

When the SP fraction was added to the S_{105} fraction, the activity was somewhat higher than SP alone. The activities of both the SP and S_{78} fractions were lost upon storage for three days in the refrigerator and in a week in the frozen state (rapid freezing in Acetone-dry ice). Neither storage under nitrogen nor addition of excess FMN affected the rate of loss. Dialysis against phosphate buffer containing EDTA and dithiothreitol, or passing through an anaerobic Bio-Gel P-2 (or P-10) column also caused a loss of activity. Reconstitution by adding lyophilized dialyzate to the dialyzed SP did not cause recovery of the original activity.

FPH. Formation activity of ammonium sulfate precipitates

Ammonium sulfate fractionation of S_{105} gave material which showed some activity. Three fractions (0—40%, 40—60% and 60% ammonium sulfate saturation) were obtained and their asorption spectra are shown in Fig. 3. The 0—40%

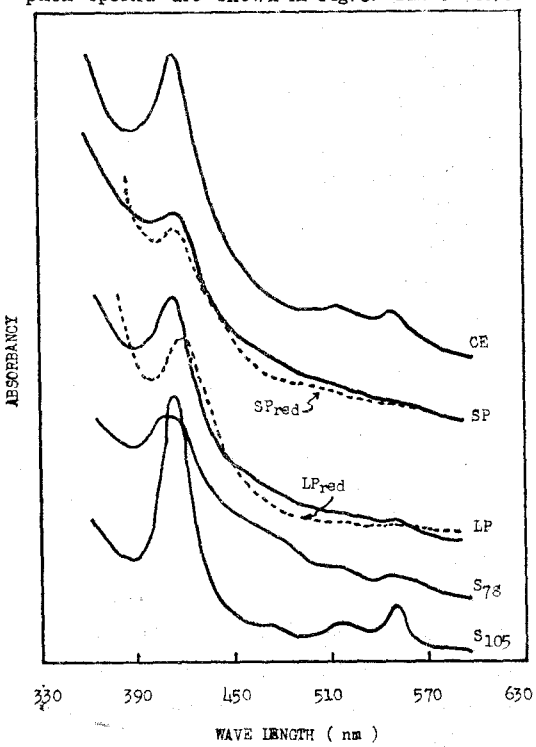


Fig. 1. Absorption Spectra of *Azotobacter vinelandii* Cell Fraction CE; Extrac, SP; Small Particle, LP; Large Particle, S_{78} and S_{105}; Supernatant at 78.500×g and 105.500×g, dotted lines; reduced by S_2O_4.

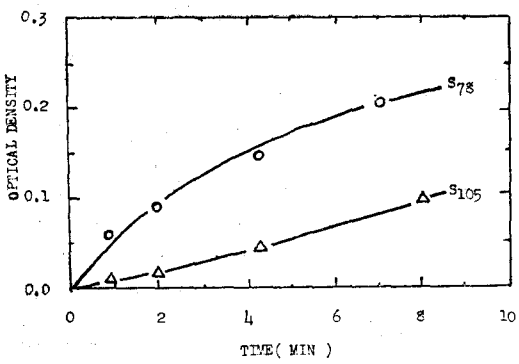


Fig. 2. Time course FPH·Formation by *Azotobacter* Cell Fractions

Table I. Specific Activity of FPH· Formation in Cell Fractions of *A. vinelandii*

Fraction	Protein (mg/ml)	Specific Activity of FPH·Formation*
CE	0.5	0.6
LP	—	—
SP	0.73	0.065
S_{78}	0.53	0.06
S_{105}	0.50	0.00

*Specitic activity is expressed as absorbance change at 580 nm per hour per mg of protein. Reaction mixture consisted of 1.0 ml of 1.92×10^{-6} M flavoprotein, 0.2 ml of cell fraction(particles were suspended in appropriate amount of phosphate buffer) and 0.2 ml of 0.1M phosphate buffer (pH=7.4).

fraction shows an absorbance maximum at 410 nm and a broad shoulder around 500 nm, typical of non-heme iron proteins. Most of the cytochromes come out in the 60% fraction and its supernatant. The supernatant of the 60% fraction was yellow in color and is known to contain the Shethna flavoprotein (1). The 60% precipitate and the crude S_{105} fraction had similar spectral properties. Also, the ammonium sulfate precipitates from S_{78} had similar spectra to those from S_{105}. The spectral changes observed upon reduction by dithionite were similar to those given by SP and LP (see Fig. 1). The 40% fraction had the highest activity among the three fractions (Table II).

Further Purification of Small Particles and 40% Ammonium Sulfate Fraction.

Since the small particle fraction (SP) and the

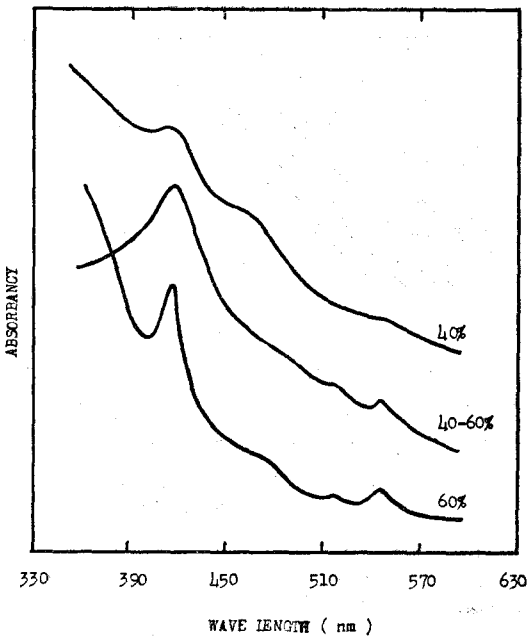


Fig. 3. Absorption Spectra of Ammonium Sulfate Fractions of S_{105}

Table II. Specific Activity of FPH· Formation in Ammonium Sulfate Fractions

Fraction	Protein (mg/ml)	Specific Activity of FPH· Formation
40%	0.262	0.005
60%	0.130	0.002
over 60%	0.220	0.000

40% ammonium sulfate precipitate had both similar spectroscopic properties and the highest activity, further purification of these active materials was carried out by DEAE column chromatography. Before application on the DEAE column, the SP fraction was dissolved in deaerated phosphate buffer, or the S_{78} fraction was used as the SP source. The ammonium sulfate precipitate was dialyzed against phosphate buffer containing EDTA (1×10^{-3}M) and dithiothreitol (1×10^{-3}M) or salt was eliminated through Biogel P-2 column which was equilibrated with the same suffer. Eluates from the Biogel column were concentrated by Diaflo under N_2 pressure, or by Aquacide II.

a) First DEAE column chromatography

The small particle fraction dissolved in buffer, or the S_{78} fraction, was treated with protamine sulfate to eliminate nucleic acid, and was applied

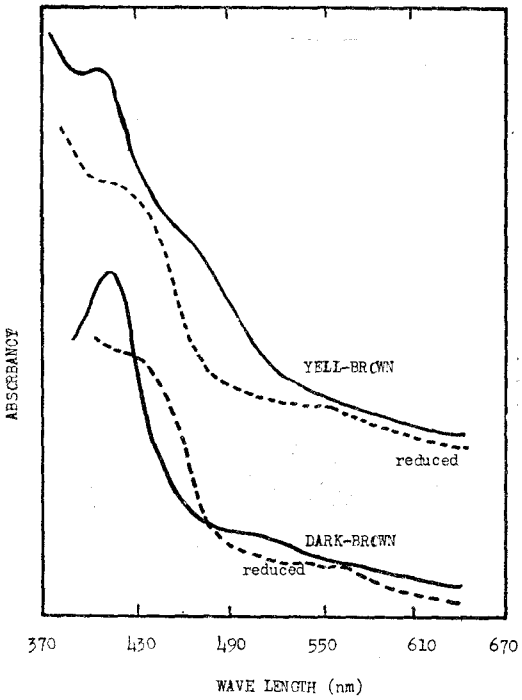


Fig. 4. Absorption Spectra of Yellowish brown and Dark brown bands on the first DEAE Chromatograph Dotted lines are Reduced Spectra by $S_2O_4^=$(dithionite).

on the DEAE column, which was previously equilibrated with deaerated 0.025M phosphate buffer (pH=7.4) and eluted stepwise with 0.025, 0.05, 0.1 and 0.2M phosphate buffer.

All phosphate buffers used contained EDTA and dithiothreitol. A 40% ammonium sulfate precipitate was also chromatographed in the Insame way, after elimination of ammonium sulfate. In spite of the different sample preparation, the chromatographic patterns in the DEAE column were essentially the same; a cytochrome band was eluted with the initial buffer together with a bluish fluorescent material, followed by a yellow fluorescent band and another yellowish-brown band with 0.1M phosphate buffer; a dark brown band eluted with 0.2M phosphate buffer while the Shethna flavoprotein, as a blue-green band, remained on the top of the column. The absorption spectra of the yellowish brown band and the dark brown-band are shown in Figure 4.

The yellow band which eluted just after the cytochrome band proved to be free FMN, showing a typical flavin absorption spectrum and the same Rf value as FMN on thin layer chromatography, and giving neither a Buiret reaction nor protein staining by Ponceau S(p-sulfobenzene-azo-0-sulfobenzene azo-naphthol-3,6-sulfonic acid sodium salt in 3% trichloroacetic acid solution) on an electrophoretic polyacetate strip. This flavin band sometimes eluted together with the yellow-brown band, depending upon the amount of eluant used. As will be shown below, the yellow-brown band eluted with 0.1M phosphate buffer was the most active material as regards FPH· formation in the presence of NADH, which was required as an electron donor substrate.

b) Second DEAE Column Chromatography

The yellow-brown bands from the 40% ammonium sulfate fraction of S_{105} and the S_{78} fraction were identical. Because of the relatively high activity in FPH· formation, the yellow-brown band was rechromatographed on a DE-32 column, which was equilibrated previously with 0.05M tris buffer. The column was developed by a gradient elution using 500 ml of 0.1 and 0.5M sodium chloride in 0.05M TRIS buffer with a flow rate of 0.42 ml per minute using a proportioning pump. The pattern obtained is shown in Fig. 5-A 0.1M Phosphate buffer (pH=7.8] containing EDTA and dithiothreitol (1×10^{-3}M) used for both equilibration and elution, also gave good separation (Fig. 5-B). Two proteins were obtained, a yellow fluorescent protein (protein I) and a brown protein (protein II) which gave single bands upon electrophoresis. Absorption spectra are shown in Fig. 6. Fluorescence excitation and emission spectra of protein Iare shown in Fig. 7. It is apparent that a flavin chromophore is present.

FPH· Formation Activity of Purified Proteins

Since the purified proteins were so unstable that denaturation easily occurred within several hours even at 0°C, the preparations were kept frozen until use and reactions were carried out in the presence of EDTA and dithiothreitol. The two purified proteins were able to form FPH·, however, only in the presence of added NADH,

The specific activities are shown in Table III. It is apparent that a considerable increase in specific activity has been achieved, although FPH· formation was not proportional to the concentration of protein.

NADH Dehydrogenase and Succinate Dehydrogenase Activities

NADH is known to serve as a primary electron donor in *Azotobater vinelandii*.[8,9] Since NADH was required for FPH· formation by proteins I and II and since the absorption spectrum of protein I resembles that of succinate dehydrogenase (10) and the small particle fraction that of NADH dehydrogenase (11), the distributions of these enzyme activities in the cell fractions were measured (Table IV), along with the NADH oxidase activity.

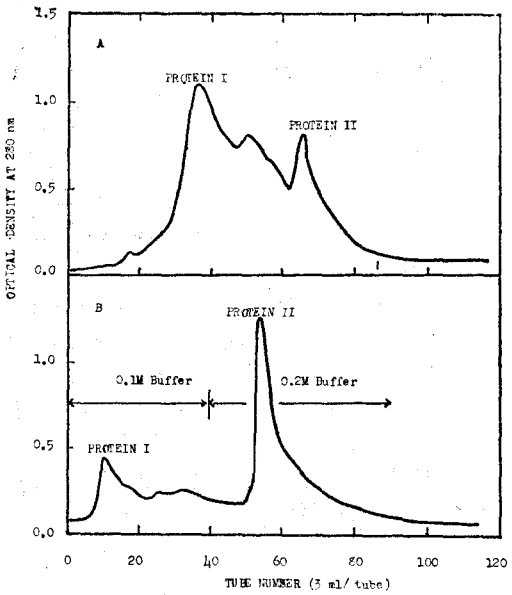


Fig. 5. Elution Pattern of Yellow-Brown Band onnDE-32 Column. A : Gradient elution, B : Stepwise elution.

Table III. Effect of Concentration of Purified Proteins on FPH· Formation

a) Protein I

Protein I (mg/ml)	Conc. Ratio	FPH·/hr.	FPH·*/mg./hr.
0.057	1	0.04	0.71
0.114	2	0.06	0.5
0.171	3	0.04	0.23

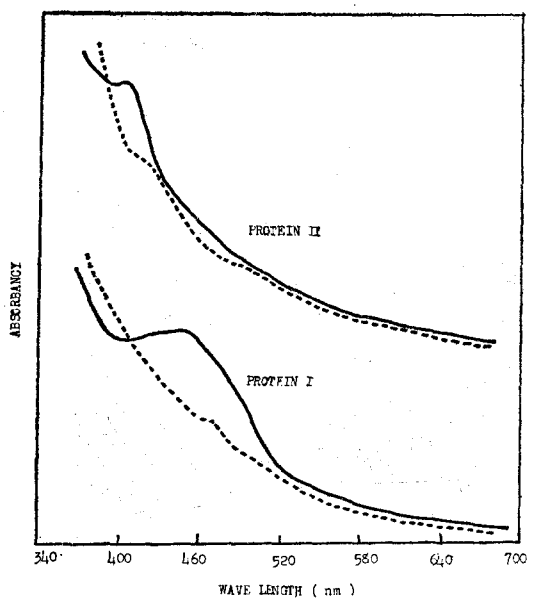


Fig. 6. Absorption Spectra of Purified Proteins Reduced by S_2O_4

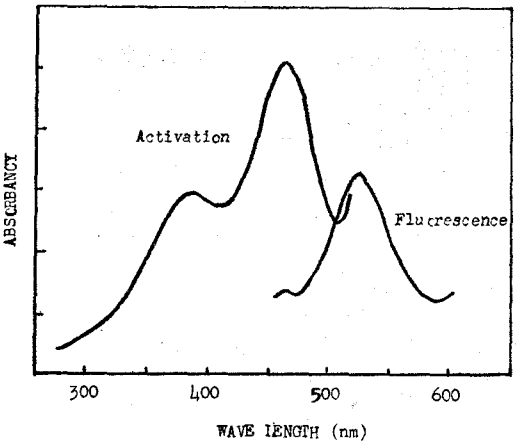


Fig. 7. Fluorescence Exitation and Emission Spectra of Protein I.

b) Protein II

Protein II (mg/ml)	Conc. Ratio	FPH·/hr.	FPH·*/mg. hr.
0.0131	1	0.060	4.62
0.0262	2	0.070	2.70
0.0437	3.3	0.050	1.10
0.131	10	0.074	0.57

*OD change at 580 nm. per hour per mg of protein.

Table IV. Distribution of Succinic Acid and NADH Dehydrogenases in *A. vinelandii* Cell Fractions

Fraction	NADH Dehydrogenase[1]	NADH Oxidase	Succinate Dehydrogenase[2]	FPH· Reduction Activity[3]
Large Particle	—	4.16	9.99	—
Small Particle	0.1010	1.30	1.63	0.065
S_{78}	0.007	12.8	2.45	0.060
S_{105}	0.007	0.0	0.476	low
SP-S_{105}*	0.067	1.08	10.25	—
40% Fraction	—	0.70	1.36	0.05**
60% Fraction	—	0.16	0.18	0.02**

1) expressed as OD change at 340 nm per minute per mg of protein at 30°C; reaction mixture consisted of 0.2 ml of 1.3×10^{-3}M NADH, 0.1 ml of enzyme solution and 0.5 ml of 0.025M phosphate buffer.
2) Expressed as OD change at 420 nm per minute per mg of protein at 30°C; reaction mixture consisted of 0.1 ml of 75 mM $K_3Fe(CN)_6$, 0.1 ml of 3% Bovine Serum Albumin, 0.3 ml of 0.04 M succinate (pH=7.6), 0.1 ml of 0.03M KCN (neutralized), 0.1 ml 30 mM EDTA (pH=7.6) and 1ml of 0.3M phosphate buffer (pH=7.6).
3) See Table I and II. NADH or succinate alone do not reduce FP.

Table IV (continued)

* Slime-like layer above SP on centrifugation at 105,500×g.

** Reaction was carried out at different conditions than others.

The enzyme activities measured were localized mostly in the particle fractions. A correlation seems to exist between the NADH oxidase and dehydrogenase activities and FPH · formation. Thus, the SP fraction had the highest NADH dehydrogenase activity and the S_{78} fraction the highest NADH oxidase activity. Both of these fractions possess appreciable FPH· formation activity. A similar correlation exists between the 40% and 60% ammonium sulfate fractions.

Addition of NADH to an old preparation of crude extract, which had no activity for FPH· formation, but still had abundant NADH oxidase activity, did not generate FPH·formation activity.

Free Flavin as a Possible Substrate for FPH·

Inasmuch as free FMN was found during the chromatography of the *Azotobacter* extract, we felt it of interest to see whether or not reduced flavins can serve as a source of electrons for the Shethna flavoprotein. Addition of NADH and FMN to a solution of the Shethna protein resulted in the slow formation of FPH·. All of the crude fractions from *Azotobacter* accelerated the reaction (Table V). It was observed that the absorbancy decrease at 450 nm occurred prior to the in crease at 580 nm (absorption maxima of free radical flavoprotein). This is shown in Fig. 8. The decrease in absorbancy at 450 nm shows that reduction of FMN is occurring.

When NADH and FMN react in equimolar concentrations under anaerobic conditions, FMN is slowly reduced. Addition of various cell fractions to the above reaction mixture accelerated this reaction (Table VI and Figure 9). The most active fraction is S_{78}. This correlates with the high NADH oxidase activity of this fraction. In order to ascertain whether $FMNH_2$ can reduce the Shethna flavoprotein to its radical form, FMN was photoreduced in the presence of EDTA in the side-arm of a Thunburg tube. The photoreduced FMN (0.6 ml of 1.9×10^{-5}M) was transferred to the main portion of the tube into a flavoprotein solution(0.3 ml of 7.5×10^{-5}M) containing 0.05 ml of 0.1M EDTA and 0.2 ml of 0.3M phosphate buffer (pH =7.8). In the dark, FPH formation occurred only to about 5% of maximum. When the solution was exposed to room light, under which $FMNH_2$ was easily formed, a 15-fold increase in FPH· was observed. Thus, FPH· forma-

iton depends upon a continually-renewed supply of $FMNH_2$. If a molar excess of FMN was present, 100% of the flavoprotein could be reduced to the radical form by irradiation.

Succinic acid or TPNH in the presence of cell extracts and FMN did not cause FPH·formation. FMN showed a higher activity for FPH·formation in a mixture of crude extract and flavoprotein, than did riboflavin or FAD(Table VII). However, there was *no* specificity in the reduction of free flavin by NADH in the presence of cell fraction, S_{105}.

Table V. FPH· Formation Activities by Cell Extracts in the Presence of FMN and NADH

Fraction	Protein (mg/ml)	(initial) OD_{580}/min.	Spec. Act.
LP	0.0025	0.0021	0.84
SP	0.0033	0.0017	0.52
S_{78}	0.0038	0.0032	0.85
S_{105}	0.0034	0.0032	0.94
none	—	0.0013	—

Table VI. Catalysis of FMN and NADH Reaction by Cell Fractions

Cell Fraction	$FMNH_2$ Formation Activity*
LP	2.4
S_{78}	6.8
SP	1.2
S_{105}	4.8

* Expressed as absorbancy change at 450 nm for 5 min per mg of protein; reaction carried out in Thunburg cuvette containing 1.2 ml of 2.12×10^{-4}M FMN, 0.1ml of cell fraction and 0.4ml of 0.1M phosphate buffer(pH=7.6) at room temperature.

Table VII. Flavin Specificity with Crude Extract for FPH· Formation

Reaction	Specific Activities
CE	0.014
CE-FMN*	0.09
CE-FAD*	0.018
CE-Riboflavin*	0.030

* Reaction mixture contained 0.2 ml of crude extract, 0.7 ml of 1.6×10^{-5}M flavoprotein and 0.4ml of 0.1M phosphate buffer (pH =7.4). The crude extract was incubated with flavin for 2 hours prior to reaction.

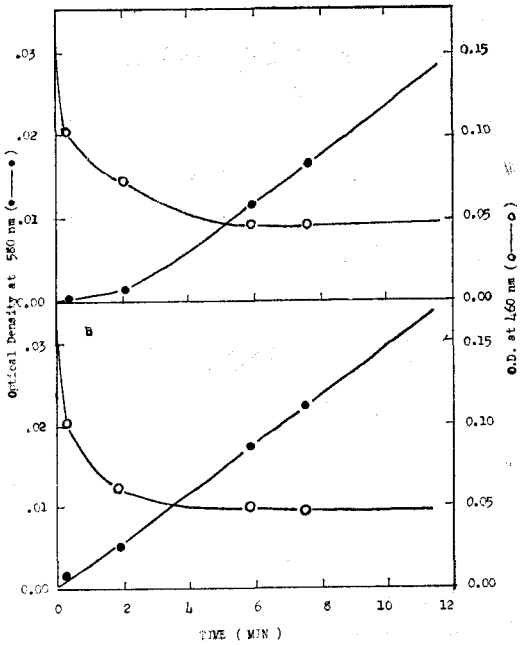


Fig. 8. Time Course Absorbance Change at 450 nm (Shethna protein's absorbancy max.) and 580 nm (half reduced Shethna proteins's absorbancy max.).

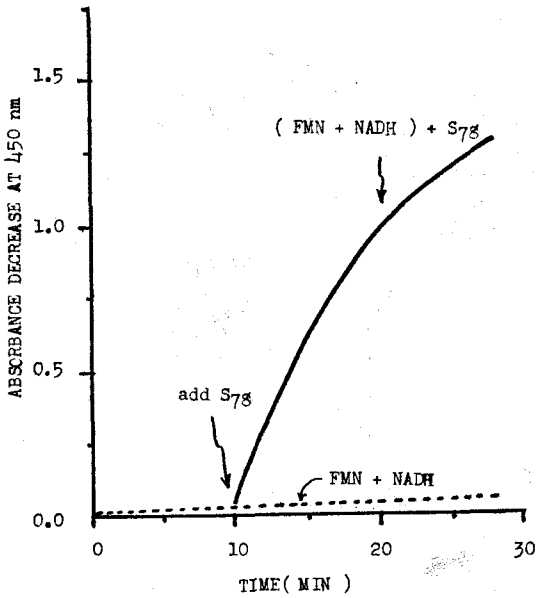


Fig. 9. Catalysis of FMN and NADH Reaction by Cell Fraction (S_{78})

On the basis of the above results, it is possible that flavin is reduced by NADH in the presence of NADH dehydrogenase or NADH oxidase in *Azotobacter*, and then the Shethna flavoprotein accepts electrons from the reduced flavin. However it is unlikely that flavins exist in the cell as free flavin but rather as a loosely bound flavoprotein, which may be easily dissociable. To test this possibility, difference spectra of FMN dissolved in the S_{78} fraction vs. FMN and S_{78} fraction in reference cells under *aerobic* conditions were measured. These show the formation of a very stable reduced flavin (Fig. 10). Flavin was rapidly reduced and stable for several hours.

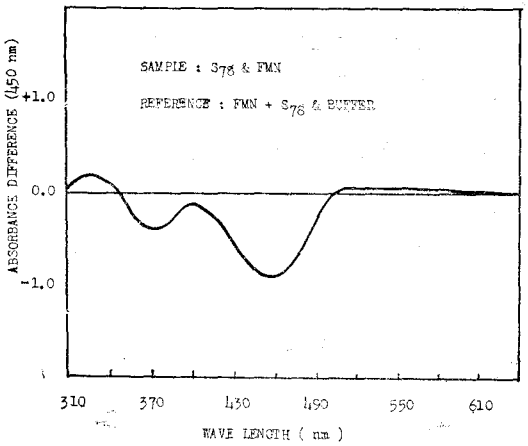


Fig. 10. Difference Spectra of FMN dissolved in S_{78} fraction vs. FMN and S_{78} fraction

CONCLUSION

The aim of these experiments was to elucidate the factor(s) that function in the transfer of electrons from substrate to the Shethna flavoprotein in *Azotobacter*. A fluorescent flavoprotein (protein I) and a brown protein (protein II) were the most active proteins which were isolated in purified form. The activity of these materials, however, was substantially decreased during the purification procedure and was completely lost upon storage. A decrease of activity upon storage was also observed in the crude cell extract. In all cases, the FPH·formation reaction was slow (half-time 5—10 hours). Whether this means that the reaction is actually non-enzymatic, or whether it is just reflection of an inherent lack of reactivity of the Shethna protein, is not clear. Since the possibility exists for electron transfer from flavoprotein to cytochrome, amytal (a cytochrome inhibitor) was added to the reaction mixture, but no change in rate was observed. Also, both reduced and oxidized cytochrome[12], prepared from *Azotobacter*, did not have any effecton FPH· formation. These results exclude the possible involvement of a cytochrome system in FPH· formation

The EPR signal in the crude extract was diminished by $K_3Fe(CN)_6$ the signal gradually reappeared after 2 hours suggesting that abundant electron donor substrates exist in the crude extract. This could be NADH, although NADH itself can not reduce the flavoprotein to the radical form. The presence of FMN with NADH enhanced the rate of FPH·formation. The reaction of FMN and NADH was found to be catalyzed by various cell fractions. $FMNH_2$ itself, easily reduced the flavoprotein into its half-reduced form. Because of the slower reaction rate of $FMNH_2+FP \rightarrow FPH\cdot+FMN$ than $FMN+NADH \rightarrow FMNH_2$, accumulation of $FMNH_2$ occurred which subsequently caused FPH· formation. A continuous electron supply to form $FMNH_2$ was necessary to complete the FPH· formation reaction. The photoreduction of FMN in the presence of EDTA gave the same result as with NADH as electron donor. No catalysis of the reaction of FPH·formation by $FMNH_2$ was found among the proteins and cell fractions thus far tested.

The SP fraction seems to be an aggregate. It was adsorbed as a dark brown band on a DEAE column and did not separate into well-defined fractions upon elution. Also dilution of a preparation of SP caused deaggregation into a soluble material. The SP fraction seems to be very closely related to protein I.

요 약

*Azotobacter vinelandi*의 細胞抽出物들이 Shethna flavoprotein의 free radical型으로의 電子傳達機構에 관하여 硏究하였다. 電子傳達에 關與度가 높은 蛋白質로 黃色螢光性蛋白質(protein Ⅰ)과 褐色蛋白質(protein Ⅱ)을 精製하였고 이들은 N_2 氣壓下 또는 aceton-dry ice 凍結저장하에서도 쉽게

失活되었고 反應速度 역시 너무 완만하여 生體內 反應이기에는 의문점을 보였다. 한편 細胞抽出物中의 FMN은 NADH에 依하여 환원이 쉽게 이루어졌으며, 환원형 $FMNH_2$는 非酵素的으로 Shethna flavoprotein의 free radical을 形成시켰으며, 酵素的反應速度보다 15倍의 높은 反應速度를 보였다. 비록 FMN이 生體內에서 他단백질과 非結合型으로 存在하지 않는다해도 FMN의 電子電達體의 可能性을 提示하였다.

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