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SOME PROPERTIES OF AN NADH–BENZYL VIOLOGEN REDUCTASE FROM *AZOTOBACTER VINELANDII*

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SUMMARY

1. NADH–benzyl viologen reductase, solubilized by acetone extraction, was purified about 10-fold from small particles of *Azotobacter vinelandii*.

2. The purified enzyme preparation was free from hydrogenase activity. Either NADH or NADPH served as an electron donor for the reduction of benzyl viologen. This reaction is reversible.

3. The essential thiol groups of the enzyme are protected since they do not react with *N*-ethylmaleimide and *p*-chloromercuribenzoate inhibits only after it has been preincubated with the enzyme.

INTRODUCTION

A benzyl viologen reductase, which requires NADH as an electron donor, has been found in *Azotobacter vinelandii*[1,2]. For some bacteria[3], mainly anaerobes, reduced viologen dye has been shown to be the substrate for the evolution of hydrogen gas by hydrogenase but *A. vinelandii* does not exhibit this kind of enzymic activity. Recently, BULEN, BURNS AND LECOMTE[4] found that small particles of *A. vinelandii* require ATP for hydrogen evolution when sodium dithionite is the electron donor. They also suggested that there are two different types of hydrogenases in the small particles; one which evolved hydrogen from $Na_2S_2O_4$ in the presence of ATP, and the other which catalyzed the uptake of the gas[5]. SHETHNA, WILSON AND BEINERT[6] purified a non-haem iron protein from Azotobacter extracts and we find that it reduces benzyl viologen when NADH is the electron donor. Some of the properties of benzyl viologen reductase extracted with acetone and purified about 10-fold from small particles of *A. vinelandii* are described.

MATERIALS AND METHODS

Culture of the bacterium

The culture of *A. vinelandii* (O) and the preparation of small particles have been reported in a previous paper[1]. Particles (P6) collected between 100000 × *g* and 144000 × *g* for 6 h were used[1].

Abbreviation: PCMB, *p*-chloromercuribenzoate.

Reagents and preparation

NAD^+, NADH, $NADP^+$ and NADPH were obtained from Calbiochem, Calif., U.S.A.; FMN, FAD, GSH and mammalian cytochrome *c* were products of Sigma Chemical Co., St. Louis, Mo., U.S.A.; benzyl viologen and methyl viologen were obtained from British Drug Houses, Poole, England. Oxygen-free nitrogen, hydrogen and argon gases were purchased from the Commonwealth Industrial Gas Pty, Adelaide. Sephadex was obtained from Pharmacia, Sweden; DEAE-cellulose (DE 11) from Whatman Co., England, and Biogel HT from Bio-Rad Laboratory, Calif., U.S.A. Ferredoxin was prepared from *Clostridium pasteurianum* according to the method of MORTENSON[7] and cytochromes c_4 and c_5 from *A. vinelandii* by the method of NEUMANN AND BURRIS[8]. Nitrate reductase was prepared from *Micrococcus denitrificans* as described in a previous paper[2]. Protein was determined according to the procedure of LOWRY *et al.*[9]. Nitrate reductase activity was measured by the method of FEWSON AND NICHOLAS[10]. NAD^+ and $NADP^+$ were determined in an Eppendorf fluoriphotometer as described by LOWRY, ROBERTS AND KAPPHAHN[11]. Iron was determined by the method of FREDRICKS AND STADTMAN[12]. The enzyme fractions were dialyzed in Visking cellulose tubing. All spectra were determined in the Unicam SP800 spectrophotometer. The enzyme fractions were prepared in the cold room at 4° and small particles separated in the Spinco ultracentrifuge (model L). The average weight molecular weights of the proteins were determined in the Spinco analytical ultracentrifuge (model E).

Assay of benzyl viologen reductase activity

The following overall enzyme reduction of benzyl viologen was studied:

$$\text{NADH} + \text{benzyl viologen} \leftrightharpoons NAD^+ + \text{reduced benzyl viologen}$$

The enzyme activity was determined by measuring NAD^+ formation fluorimetrically or the production of reduced benzyl viologen colorimetrically. Another method used to measure the enzyme was to couple the reduced benzyl viologen produced in the reaction to a purified nitrate reductase enzyme from *M. denitrificans*[2].

The basal assay mixture in Thunberg tubes contained: 4 μmoles NADH, 1 μmole benzyl viologen in 2.3 ml of 0.05 M phosphate buffer (pH 7.5) (unless otherwise stated, the phosphate buffer is at this concentration and pH in the section that follows), in the tube, and between 50 and 200 μg enzyme protein and 10 μmoles GSH in 0.2 ml phosphate buffer in the side arm. The Thunberg tubes were evacuated and flushed 3 times with oxygen-free nitrogen gas and then filled with nitrogen. After a 10-min preincubation of the enzyme with GSH at 30° in a reciprocating water bath, the reaction was allowed to proceed for a predetermined time (between 5 and 15 min, depending on the enzyme activity), and then the NAD^+ formed was analysed.

When reduced benzyl viologen was measured, all the components in the reaction mixture were increased 3-fold. After a given time, the tube was fitted directly into a Hilger photometer and the absorbance was measured using filter 70. A standard curve for reduced benzyl viologen was made by reducing various concentrations of dye with sodium dithionite.

A control tube containing a boiled enzyme was also included in all the experiments. The activity is expressed in μmoles of either NAD^+ formed or reduced benzyl viologen produced.

RESULTS

Solubilization and purification of the enzyme from small particles

Small particles suspended in an equal volume of cold butanol were stirred vigorously with a magnetic stirrer in a cold room at 4° for 1 h. After centrifuging at 144000 × *g* for 6 h, the water phase was collected and dialyzed overnight against phosphate buffer. Another method used to extract the enzyme was to treat small particles with cold 50 % (v/v) acetone–water at 4° as for the butanol extraction. The supernatant fraction collected, by centrifuging as described previously, was brought to 67 % (v/v) with respect to acetone. The mixture was centrifuged again and the resultant precipitate was dissolved in phosphate buffer and dialyzed overnight. The dialyzed preparation was centrifuged at 10000 × *g* for 2 h; the supernatant contained the enzyme activity. Details of these treatments are given in Table I. The acetone treatment was more efficient than the butanol procedure in solubilizing the enzyme from small particles.

TABLE I

SOLUBILIZATION OF NADH–BENZYL VIOLOGEN REDUCTASE FROM SMALL PARTICLES

	Total protein (mg)	*Total activity* (μmoles NO_2^- per 15 min)*	*Specific activity*
Small particles (P6)	384	451	1.2
Butanol treatment	78	32	0.4
Acetone treatment	92	154	1.7

* Reduced benzyl viologen formed measured by coupling it to a purified nitrate reductase from *M. denitrificans* and determining nitrite formed (see MATERIALS AND METHODS). Reaction mixture: NADH 2 μmoles, benzyl viologen 1 μmole, KNO_3 1 μmole, enzyme protein 100 μg and Micrococcus nitrate reductase 200 μg in 2.5 ml phosphate buffer. Small particles (P6), collected between 100000 × *g* and 144000 × *g* for 6 h, contained about 10 mg protein per ml in 0.05 M phosphate buffer (pH 7.5), were treated with butanol or acetone as described in MATERIALS AND METHODS.

Details of enzyme purification are given in Table II. About 80 mg (protein) P6 particles suspended in 80 ml phosphate buffer were treated with acetone to extract the enzyme as described previously. The solubilized enzyme (20 ml, Fraction II) was passed through a Sephadex G-100 column (2.5 cm × 25 cm). When the column was eluted with water a brown band was collected (20 ml, Fraction III). This fraction was loaded onto a DEAE-cellulose column (1.5 cm × 12 cm) and after washing with water the column was developed with 0.1 M Tris–HCl buffer (pH 8.0). A red fraction, which was mainly cytochrome of the *c* type, was eluted. The Tris buffer was then increased to 0.4 M (pH 8.0) to elute the brown fraction (20 ml, Fraction IV). Solid $(NH_4)_2SO_4$ was added to Fraction IV to bring it to 65 % satn. The precipitate collected by centrifuging at 5000 × *g* for 10 min was dissolved in the phosphate buffer and dialyzed against the buffer overnight (5 ml, Fraction V). Fraction V was rechromatographed on a DEAE-cellulose column as for Fraction IV.

The eluate, precipitated by adding $(NH_4)_2SO_4$ to 35 % satn., was centrifuged and the residue treated as for Fraction V (3 ml, Fraction VI).

TABLE II

PURIFICATION OF THE NADH–BENZYL VIOLOGEN REDUCTASE

The assay system was as described in MATERIALS AND METHODS. Reaction time, 5 min.

Fraction	*Total vol. (ml)*	*Total activity (μmoles NAD^+ per 5 min)*	*Total protein (mg)*	*Specific activity (μmoles NAD^+ per mg protein per 5 min)*
I. Small particles (P6) in 0.05 M phosphate buffer (pH 7.5)	80	2152	920	2.3
II. Enzyme extracted by acetone–water (50%, v/v) and precipitated increasing to 67% acetone. Precipitate dissolved in phosphate buffer and dialysed overnight	20	756	216	3.5
III. Fraction II was added to a Sephadex G-100 column, eluted with water	20	700	124	5.6
IV. Fraction III was added to a DEAE-cellulose column, washed with 0.1 M Tris–HCl buffer (pH 8.0), then brown band was eluted by 0.4 M Tris buffer (pH 8.0)	20	550	68	8.1
V. Enzyme was precipitated by $(NH_4)_2SO_4$ (0~60% satn.) from Fraction IV dissolved in phosphate buffer, then dialysed overnight	5	420	30	14.0
VI. After rechromatography of Fraction V on DEAE-cellulose column, the enzyme was precipitated by $(NH_4)_2SO_4$ (0~35% satn.), then dissolved in phosphate buffer and dialysed overnight	3	275	17	18.2

Further purification of the enzyme was not achieved with either calcium phosphate gel, alumina $C\gamma$ gel or with a Biogel column. The enzyme activity of Fraction VI was split into two different proteins by Biogel HT column chromatography. The first fraction was eluted from the column with 0.05 M phosphate buffer (pH 7.5) and the second with 0.1 M pyrophosphate (pH 8.0). The enzymic activity was considerably reduced in both fractions and it was not reconstituted when they were mixed.

Spectra of the purified enzyme

An absorption spectrum of Fraction VI had a maximum absorption due to protein at 280 mμ and a small shoulder at 290 mμ. A small absorption was detected in the Soret region at 410 mμ. The $A_{280\,m\mu}/A_{260\,m\mu}$ ratio was about 1.43. The addition of sodium dithionite to this preparation did not alter the spectrum.

Isoelectric point

The protein was precipitated by acetic acid at pH 4.2 and redissolved at pH 3.8 by further addition of acetic acid. This indicates that the isoelectric point of the preparation was at approximately pH 4.

Starch-gel electrophoresis

Starch-gel electrophoresis of Fraction VI in 8 M urea and 1 M acetic acid (pH 3), 400 V for 5 h, showed about 6 protein bands as compared with 14 bands in the original small particles. Thus the faster running components were removed on purification of the enzyme.

Weight average molecular weight of Fraction VI

The weight average molecular weight of this preparation was 68000 obtained by the approach to sedimentation equilibrium method in the Spinco analytical ultracentrifuge as described by SCHACHMAN[13]. The sample contained 6.5 mg protein per ml of 0.05 M phosphate buffer (pH 7.5).

pH and temperature

The optimum pH of the reaction was approx. 8.5. A temperature–activity curve was plotted; above 40°, there was a rapid increase in activity and the Q_{10} between 40° and 50° was about 3.3 after a 2-min reaction period.

Substrate specificity of the enzyme

The partially purified enzyme preparation (Fraction VI) did not catalyse the reduction of benzyl viologen by hydrogen gas, although small particles (Fraction I) showed this hydrogenase activity. The effect of hydrogen and reduced and oxidised forms of various electron donors are listed in Table III. The results show that there are two different enzymes in the small particles; a hydrogenase which reduces benzyl viologen in the presence of hydrogen gas, and a NADH– or NADPH–benzyl viologen oxidoreductase. Methyl viologen did not substitute for benzyl viologen in these enzyme reactions. Dihydrolipoic acid did not replace either NADH or NADPH as an electron donor. Neither lipoate dehydrogenase nor transhydrogenase activity was detected in Fraction VI.

TABLE III

EFFECT OF NAD^+, $NADP^+$ AND THEIR REDUCED FORMS ON HYDROGENASE ACTIVITY IN FRACTIONS I AND VI

Basal reaction mixture in Thunberg tubes contained: tube: 3 μmoles benzyl viologen and other reagents as listed each at 12 μmoles; side arm: 10 μmoles GSH, Fraction I or VI 100 μg protein. The final volume was adjusted to 7.5 ml with phosphate buffer. The tubes were evacuated and filled with hydrogen gas; this procedure was repeated 3 times.

Fraction	*Nucleotide additions*	*Time (min) required to reduce 0.1 μmole benzyl viologen*
I. P6 particles (see MATERIALS AND METHODS)	None	20
	NAD^+	5
	$NADP^+$	5
	NADH	0.5
	NADPH	0.5
VI. Purified enzyme (see Table II)	None	*
	NAD^+	*
	$NADP^+$	*
	NADH	2
	NADPH	2

* No reduction after 180 min.

Substrate concentration

The rate of reaction was measured at various concentrations of NADH or NADPH and benzyl viologen. The results are plotted in Fig. 1 according to Lineweaver and Burk. The calculated K_m values for each substrate are as follows: $4.0 \cdot 10^{-3}$ M, NADH; $2.8 \cdot 10^{-3}$ M, NADPH; and $1.3 \cdot 10^{-3}$ M, benzyl viologen.

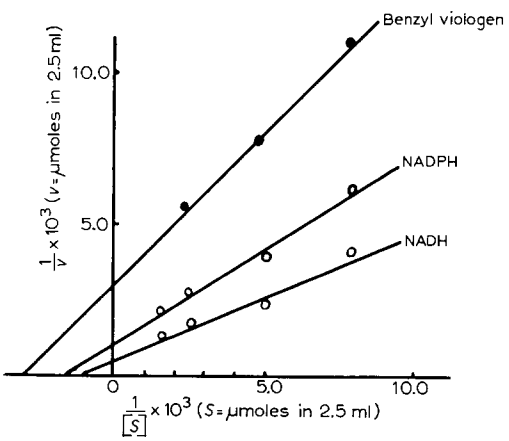

Fig. 1. Effect of substrate concentrations for Fraction VI. Reaction mixtures in Thunberg tube: tube: NADH or NADPH 1.2, 2.0, 4.0 and 6.0 μmoles, benzyl viologen 1 μmole; side arm: GSH 10 μmoles, enzyme protein 100 μg, in the total volume of 2.5 ml phosphate buffer. After incubation period of 10 min NAD^+ or $NADP^+$ formed was determined as described in MATERIALS AND METHODS. Reaction mixtures for benzyl viologen: tube: NADH 4 μmoles, benzyl viologen 2.0, 4.0 and 6.0 μmoles, respectively; side arm: as described above. NAD^+ formed was determined in the same way as described above. The calculated K_m values are $4.0 \cdot 10^{-3}$ M for NADH, $2.8 \cdot 10^{-3}$ M for NADPH and $1.3 \cdot 10^{-3}$ M for benzyl viologen.

Equilibrium point of the reaction

The equilibrium point of the enzyme reaction was determined, under the specified conditions, as a function of the ratio of reductant to oxidant. The shift of equilibrium point was followed by adding varying amounts of NADH to a fixed amount of benzyl viologen. The results in Fig. 2 show that the addition of NADH moves the equilibrium point towards a further reduction of benzyl viologen.

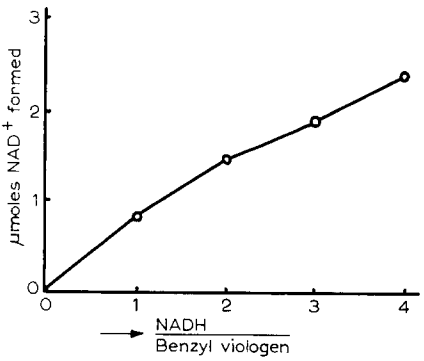

Fig. 2. Equilibrium point of the enzyme (Fraction VI) as influenced by the concentration of NADH. Reaction mixtures: tube: NADH 3, 6, 9, and 12 μmoles, benzyl viologen 3 μmoles; side arm: GSH 10 μmoles; enzyme 200 μg protein, in total volume of 7.5 ml phosphate buffer. Incubation period for 1 h.

Reversal of the reaction

The reverse reaction, *i.e.* oxidation of reduced benzyl viologen was examined by determining the reduction of added NAD^+ using the fluorimetric method. In a special Thunberg tube[14], 2.2 μmoles of benzyl viologen in 2.0 ml phosphate buffer

TABLE IV

STOICHIOMETRY OF THE ENZYME REACTION

Reaction mixture: 10 μmoles GSH, 200 μg enzyme protein and reactants as indicated, in a total volume of 7.5 ml phosphate buffer, incubated for 1 h. Equilibrium was reached after approx. 30 min. Values in μmoles.

Fraction	*Reactants*		*Products*	
	NADH	*Benzyl viologen*	*NAD^+*	*Reduced benzyl viologen*
I. P6 particles	12.0	3.0	2.8	0.9
VI. Purified enzyme	12.0	3.0	2.6	0.8
VI. Purified enzyme	3.0	3.0	1.8	0.5
	NADPH	*Benzyl viologen*	*$NADP^+$*	*Reduced benzyl viologen*
I. P6 particles	12.0	3.0	2.0	0.7
VI. Purified enzyme	12.0	3.0	2.7	0.9
VI. Purified enzyme	3.0	3.0	1.9	0.6

(pH 7.5) were reduced chemically with hydrogen gas and palladized asbestos according to the method of WALKER AND NICHOLAS[14]. The reduced benzyl viologen was then mixed with 2 μmoles of NAD^+ and 100 μg of Fraction VI in 0.5 ml of phosphate buffer under vacuum and this mixture was incubated for 1 h at 30°. A reduction of 1.4 μmoles NAD^+ was observed after this period which is approx. 63 % of the added NAD^+, assuming that reduced benzyl viologen was 90 % pure.

Stoichiometry of the enzyme reaction

The stoichiometry of the enzyme reaction was investigated by measuring the components at equilibrium. Approx. 3 moles of NADH or NADPH were consumed for every mole of benzyl viologen reduced, as shown in Table IV (Fraction I or VI). When the gas phase was hydrogen (prepared free from oxygen by passage through a deoxygenating cartridge) or argon instead of nitrogen, the results were similar. Although NADH oxidase activity was detected in air in these fractions, it was negligible under anaerobic conditions.

Product inhibition

A product inhibition was observed when a fixed amount of NAD^+ or $NADP^+$ was added to a reaction mixture containing varying amounts of either NADH or NADPH as shown in Fig. 3.

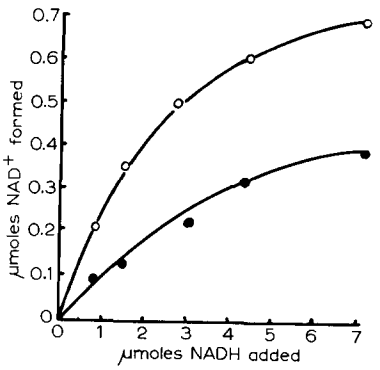

Fig. 3. Product inhibition of Fraction VI. Reaction mixtures: tube: NADH 1.4, 2.8, 4.2, 5.6 and 7.0 μmoles, benzyl viologen 1 μmole and to each tube 4 μmoles NAD^+ were added; side arm: GSH 10 μmoles and 50 μg enzyme protein. Incubation period for 10 min. The inhibition curve was derived by subtracting the amount of NAD^+ added to the reaction mixture initially from the amount present at the end of the incubation period. ○—○, control; ●—●, NAD^+ added.

Effect of adding metallic ions and electron transfer components

The following salts, each at $4 \cdot 10^{-3}$ M when added to the basic reaction mixture, had no effect on NAD^+ formation: $Fe(NH_4)_2(SO_4)_2$, $FeCl_3$, $CaCl_2$, $CuSO_4$, $MgSO_4$, $(NH_4)_6Mo_7O_{24}$ and KCN.

Mammalian cytochrome *c* and Azotobacter cytochromes c_4 and c_5, each at 100 μg, had no effect on NAD^+ formation but FMN and FAD at a final concn. of $1 \cdot 10^{-3}$ M, and a flavoprotein (0.1 ml) from *A. vinelandii*, prepared as described by SHETHNA, WILSON AND BEINERT[6], inhibited the enzyme.

Effect of thiol reagents

The addition of thiol compounds (GSH, cysteine, β-mercaptoethanol and dithiothreitol) did not usually enhance the enzyme activity, but it was markedly depressed by some –SH inhibitors. Thus *p*-chloromercuribenzoate (PCMB), iodoacetate and mercuric chloride inhibited the reaction after preincubation with Fraction VI, but this inhibition was reversed by adding GSH or cysteine. The results in Table V show that *N*-ethylmaleimide and GSSG did not inhibit the purified fraction. Although Fraction VI did not react with *N*-ethylmaleimide, the supernatant left after centrifuging a boiled sample of it (6 min at 100°) reacted with this reagent.

TABLE V

EFFECT OF THIOL REAGENTS ON THE PURIFIED ENZYME (FRACTION VI)

Reaction mixture contained in Thunberg tube: tube: NADH 4 μmoles, benzyl viologen 1 μmole; side arm: 50 μg enzyme protein and each reagent at 10 μmoles, in a total volume of 2.5 ml phosphate buffer. The enzyme and the thiol reagents were preincubated together for 10 min at 30°, prior to adding the contents of the side arm to the tube.

Reagents (*10 μmoles*)	*Enzyme activity* (*μmole NAD^+ per 10 min*)
None	0.13
GSH	0.13
PCMB	0.04
Iodoacetate	0.06
Mercuric chloride	0.05
PCMB + GSH*	0.13
Iodoacetate + GSH*	0.13
N-Ethylmaleimide	0.12
GSSG	0.13

* The enzyme was incubated with the inhibitor for 10 min, then 20 μmoles GSH added and after a further 10-min period the reaction was started.

The oxidation of Fraction VI by potassium ferricyanide or its reduction by sodium dithionite did not affect enzyme activity, but iodine treatment deactivated the system and this effect was not reversed by adding GSH.

The addition of increasing amounts of either Fraction I or VI to the basic assay mixture moved the equilibrium point as shown in Fig. 4. This effect was observed

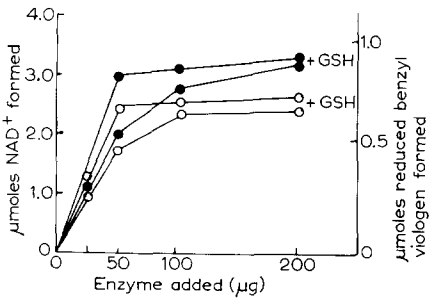

Fig. 4. The equilibrium of the enzymic reaction as influenced by adding GSH to Fraction VI. The assay mixture is as described in MATERIALS AND METHODS with and without GSH. Mixtures containing 25–200 μg enzyme in the total volume of 7.5 ml phosphate buffer were incubated for 1 h. ●—●, reduced benzyl viologen; ○—○, NAD^+.

with either NADH or NADPH with or without GSH. This suggests that a thiol stabilizer may be required for enzyme action.

Effect of iron reagents

Fractions I and VI contained 8.0 and 2.7 μg Fe per mg protein, respectively. No change in activity was observed when the enzyme was incubated with NaEDTA, α,α'-dipyridyl, 8-hydroxyquinoline and *bis-o*-phenanthroline at a final concn. of $4 \cdot 10^{-3}$ M.

When Fraction VI was treated with $Na_2S_2O_4$ followed by a dialysis against 0.05 M phosphate buffer containing 0.05 % bis-*o*-phenanthroline, the dialyzate became red, indicating the removal of ferrous iron. This treatment, however, did not depress enzyme activity. Ferredoxin either in the oxidized or reduced form had no effect on the purified enzyme.

DISCUSSION

HYNDMAN, BURRIS AND WILSON[15] observed that a cell-free preparation of *A. vinelandii* contained hydrogenase which took up hydrogen when benzyl viologen was present. Later, PECK AND GEST[3] found that Azotobacter hydrogenase, unlike *C. pasteurianum* did not evolve hydrogen from reduced methyl viologen. BURNS AND BULEN[5] studied the ATP-dependent hydrogen-evolving enzyme using $Na_2S_2O_4$ as an electron donor, since this was required for nitrogen fixation in cell-free extracts[4,16].

Ferredoxin, a non-haem iron protein, was isolated from *C. pasteurianum* by MORTENSON[7] and MORTENSON, VALENTINE AND CARNAHAN[17] and another non-haem iron complex, first identified in *A. vinelandii* by NICHOLAS *et al.*[18], has since been purified by SHETHNA, WILSON AND BEINERT[6] and SHETHNA *et al.*[19]. NAIK AND NICHOLAS[1,2] have described a NADH–benzyl viologen reductase in small particles (P6) of *A. vinelandii*. Since these particles contain hydrogenase, nitrogenase and non-haem iron protein, further work has been done to determine the relation if any, of NADH–benzyl viologen reductase enzyme to these systems.

The main difficulties associated with the purification of this enzyme included its strong affinity to other acidic proteins since they were separated only in starch gel using 8 M urea in 1 M acetic acid and a possible irreversible denaturation associated with thiol Fe which was unaffected by adding either GSH or dithiothreitol.

The thiol groups of the enzyme appear to be protected since a preincubation with PCMB is necessary for inhibition, *N*-ethylmaleimide reacts only with the heat-denatured enzyme, and thiol groups are resistant to oxidation by ferricyanide.

The precipitation of benzyl viologen reductase at its isoelectric point deactivated the enzyme and volatile sulphide was evolved. The addition of a small amount of GSH moved the equilibrium point slightly and this effect was more marked at low enzyme concentrations suggesting that thiol compounds were required to stabilize the enzyme.

The purified enzyme preparation (Fraction VI) contained 3 atoms Fe per mole protein, but the enzyme activity was not affected by inhibitors of the metal. Since ferredoxin had no effect, the role of iron in this enzyme remains to be determined. The purification procedure removed hydrogenase, transhydrogenase and lipoate dehydrogenase leaving NADH or NADPH as the sole electron donor for benzyl viologen

reduction. Since the addition of this enzyme system to small particles (P6) had no effect on either nitrogen fixation or hydrogen evolution, it is unlikely to be directly involved in these processes.

The reasons for the stoichiometry 1 mole benzyl viologen per 3 moles NADH or NADPH are obscure. Possible explanations are that residual oxygen in the Thunberg tubes may have oxidised reduced benzyl viologen and that benzyl viologen reduction by NADH might involve more than a one-electron change.

Since the enzyme activity on a Biogel column was split into two fractions and each one utilized either NADH or NADPH, it is likely that these are isoenzymes.

The enzymic reduction of benzyl viologen ($E_0' = -0.36$ V at pH 7) by NADH ($E_0' = -0.32$ V at pH 7) did not require ATP. The extent of benzyl viologen reduction was determined by the ratio of reductant to oxidant and was independent of ATP supply.

REFERENCES

1 M. S. NAIK AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 118 (1966) 195.
2 M. S. NAIK AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 131 (1967) 204.
3 H. D. PECK AND H. GEST, *J. Bacteriol.*, 71 (1956) 70.
4 W. A. BULEN, R. C. BURNS AND J. R. LECOMTE, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 532.
5 R. C. BURNS AND W. A. BULEN, *Biochim. Biophys. Acta*, 105 (1965) 437.
6 Y. I. SHETHNA, P. W. WILSON AND H. BEINERT, *Biochim. Biophys. Acta*, 113 (1966) 235.
7 L. E. MORTENSON, *Biochim. Biophys. Acta*, 81 (1964) 71.
8 N. P. NEUMANN AND R. H. BURRIS, *J. Biol. Chem.*, 234 (1959) 3286.
9 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
10 C. A. FEWSON AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 48 (1961) 208.
11 O. H. LOWRY, N. R. ROBERTS AND J. I. KAPPHAHN, *J. Biol. Chem.*, 224 (1957) 1047.
12 W. W. FREDRICKS AND E. R. STADTMAN, *J. Biol. Chem.*, 240 (1965) 4065.
13 H. K. SCHACHMAN, *Methods in Enzymology*, Vol. 4, Academic Press, New York, N.Y., 1957, p. 32.
14 G. C. WALKER AND D. J. D. NICHOLAS, *Biochim. Biophys. Acta*, 49 (1961) 350.
15 L. A. HYNDMAN, R. H. BURRIS AND P. W. WILSON, *J. Bacteriol.*, 65 (1953) 522.
16 W. A. BULEN, K. C. BURRIS AND J. R. LECOMTE, *Biochem. Biophys. Res. Commun.*, 17 (1964) 265.
17 L. E. MORTENSON, R. C. VALENTINE AND J. E. CARNAHAN, *Biochem. Biophys. Res. Commun.*, 7 (1962) 448.
18 D. J. D. NICHOLAS, P. W. WILSON, W. HEINEN, G. PALMER AND H. BEINERT, *Nature*, 196 (1962) 433.
19 Y. I. SHETHNA, P. W. WILSON, R. E. HANSEN AND H. BEINERT, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 1263.