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Reduction of viologen dyes and a non-haem iron protein by NADH in particles from *Azotobacter vinelandii*

It has been shown that small particles prepared from *Azotobacter vinelandii*, catalyse the reduction of benzyl viologen when NADH is the hydrogen donor[1]. This reaction is insensitive to inhibitors that uncouple oxidative phosphorylation, except 2,4-dinitrophenol, but the action of 2,4-dinitrophenol was found to be that of an electron acceptor and not an uncoupler[1]. The enzymic reduction of the more electronegative dye, methyl viologen by NADH was barely detectable by spectroscopic methods. In the present paper a more sensitive procedure is described to follow this reaction in the particles, by coupling the reduced methyl viologen formed, to the enzymic reduction of nitrate to nitrite. This was achieved by incorporating a purified nitrate reductase enzyme from the denitrifying bacterium, *Micrococcus denitrificans* in the reaction mixture. With this method the reduction by NADH of methyl viologen or of a non-haem iron protein preparation from *A. vinelandii* is now demonstrated. It is also shown that the viologen or the non-haem iron reducing enzyme is specific for NADH and that NADPH and dihydrolipoate act as indirect hydrogen donors for the reaction *via* a transhydrogenase enzyme.

Nitrate reductase

This enzyme from *M. denitrificans* is associated with particles[2]. A purified nitrate reductase was prepared from these particles by extracting them with deoxycholate and separating the enzyme on a DEAE-cellulose column[3]. The purified enzyme utilizes either reduced benzyl viologen or reduced methyl viologen as a hydrogen donor but NADH and NADPH are ineffective.

Non-haem iron protein

This was prepared (50% pure, 7 mg protein/ml) from *A. vinelandii* by the method described by SHETHNA, WILSON AND BEINERT[4].

The preparation of small particles (P6) from Azotobacter, collected between 100000 and 144000 $\times g$, has been described[1].

Enzyme assay

Reduction of benzyl viologen was followed anaerobically at 610 mμ, in cuvettes fitted with Thunberg attachments as described previously[1].

Specificity of NADH for the reduction of the viologen dyes

It was previously reported[1] that when NADPH was used in place of NADH, there was a delay of about 3 min before benzyl viologen reduction commenced. This lag period was abolished after preincubating NADPH and NAD^+ for 15 min with the Azotobacter particles (P6), prior to adding benzyl viologen from the side arm. An active NADPH:NAD^+ transhydrogenase (EC 1.6.1.1) was found in the particles; thus using the lactate dehydrogenase method to determine NADH formed in the transhydrogenase reaction the following result was obtained: 0.24 μmole NADH formed/20 min per mg protein. It was also established that NADH produced by the

transhydrogenase enzyme was preferentially oxidised by the NADH-specific lactate dehydrogenase enzyme and under these conditions the reduction of benzyl viologen did not take place.

Equivalent quantities of dihydrolipoic acid (E_0' at pH 7 = —0.32 V) did not replace NADH as the hydrogen donor in the reaction. When 2 μmoles of NAD^+ were added along with dihydrolipoate, however, the benzyl viologen was reduced. This latter reaction was completely inhibited by 10^{-3} M arsenite. These results indicate that dihydrolipoic dehydrogenase (EC 1.6.4.3) was also present in the particles. This was confirmed in an independent assay in which the reduction of NAD^+ was followed at 340 mμ in the presence of added dihydrolipoate. Thus 0.14 μmole of NADH were formed/mg protein (P6) per 10 min.

NADH-viologen reductase coupled to nitrate reductase from M. denitrificans

The reduction of the more electronegative dye, methyl viologen was followed by coupling it to a nitrate reductase from *M. denitrificans*. The following mixture, in a final volume of 2 ml in Thunberg tubes incubated anaerobically at 30° for 15 min contained in μmoles: phosphate buffer (pH 7.5), 50; methyl viologen, 1; NADH, 2; KNO_3, 1; nitrate reductase from *M. denitrificans* (0.1 mg protein) and in the side arm, the Azotobacter particles, P6 (0.5 mg protein). Nitrite was assayed as described previously[5]. When benzyl viologen was used instead of methyl viologen, a fourth of the Azotobacter particles was enough to produce an equivalent amount of nitrite. The reaction was extremely sensitive to *p*-chloromercuribenzoate, since $2 \cdot 10^{-5}$ M inhibited the reaction completely with either viologen. At these concentrations *p*-chloromercuribenzoate had no effect on the nitrate reductase enzyme. The inhibition was completely reversed by adding glutathione or cysteine hydrochloride each at 10^{-3} M.

The 2,4-dinitrophenol effect

2,4-Dinitrophenol at 10^{-4} M inhibited nitrite production in the coupled reaction. This was due to the chemical oxidation of reduced benzyl viologen or reduced methyl viologen, formed in the reaction, by 2,4-dinitrophenol. The 2,4-dinitrophenol effect was overcome by generating NADH with ethanol and alcohol dehydrogenase, so that adequate reducing power was available for the reduction of both 2,4-dinitrophenol and nitrate (Table I). When reduced benzyl viologen was produced by a chemical reduction of the dye with H_2 gas in the presence of palladised asbestos in a special dual Thunberg tube fitted with a sinter[5,6], it reduced 2,4-dinitrophenol non-enzymically, to 2-amino-4-nitrophenol identified by spectrophotometric and by thin-layer chromatography of the authentic pure compound.

The reduction of a non-haem iron protein by NADH in Azotobacter particles

The results in Table II show that a non-haem iron protein preparation from Azotobacter[5] could replace benzyl viologen or methyl viologen as an electron carrier for the reduction of nitrate in the coupled system. The rate of the reaction was however much slower than that with either viologen. Addition of higher concentrations of the non-haem iron protein preparation inhibited the reaction but this effect was overcome by generating NADH with the alcohol dehydrogenase method.

This reaction appears to have a function similar to the flavoprotein enzyme

TABLE I

THE REVERSAL OF THE 2,4-DINITROPHENOL INHIBITION OF NADH-BENZYL VIOLOGEN AND -METHYL VIOLOGEN REDUCTASE FROM AZOTOBACTER BY GENERATING NADH WITH ALCOHOL DEHYDROGENASE

The reaction mixture in a final vol. of 2 ml in Thunberg tubes containing, in μmoles: phosphate buffer (pH 7.5), 50; benzyl viologen or methyl viologen, 1; ethanol, 50; alcohol dehydrogenase (10 μg protein); KNO_3, 1; 2,4-dinitrophenol, 0.2; nitrate reductase from Micrococcus, 0.1 mg protein; and in the side arm NAD^+, 1 and Azotobacter particles (P6) 0.4 and 0.1 mg protein with methyl viologen and benzyl viologen, respectively. The tubes were evacuated and the reaction started by tipping in the contents of the side arm. Separate tubes were incubated at 30° for each time interval as indicated. 2,4-Dinitrophenol was omitted from the control tube. Nitrite was determined as described in the text.

Incubation time (min)	*mμmoles nitrite per mg protein Azotobacter particles (P6)*	
Electron carrier	*Benzyl viologen*	*Methyl viologen*
15 (without 2,4-dinitrophenol)	4480	864
15 (with 2,4-dinitrophenol)	800	0
30 (with 2,4-dinitrophenol)	1360	60
45 (with 2,4-dinitrophenol)	2040	320
90 (with 2,4-dinitrophenol)	6040	980

TABLE II

REDUCTION OF A NON-HAEM IRON PROTEIN BY NADH WITH AZOTOBACTER PARTICLES COUPLED TO A NITRATE REDUCTASE ENZYME FROM MICROCOCCUS

The complete reaction mixture in a final vol. of 2 ml in Thunberg tubes contained in μmoles: phosphate buffer (pH 7.5), 50; KNO_3, 1; nitrate reductase from Micrococcus (0.1 mg protein); a non-haem iron protein preparation[4] (7 mg protein/ml), as indicated; Azotobacter particles (0.1 mg protein) and in the side arm, NADH, 2 and benzyl viologen or methyl viologen 1, as indicated. The tubes were evacuated and the reaction started by tipping in the contents of the side arm. Nitrite formed after 30 min incubation at 30° was determined as described in the text.

Additions to the basal reaction mixture		*NO_2^- produced (mμmoles)*
Non-haem iron protein (ml)	*Viologen dyes*	
0	—	0
0.2	—	6.2
0.2 (omit nitrate reductase)	—	0
0.4	—	7.0
0.6	—	5.0
1.0	—	0
1.0 (with NADH-generating system)*	—	9.7
0	benzyl viologen	76.0
0	methyl viologen	25.1

* NADH-generating system; ethanol 50 μmoles and alcohol dehydrogenase (10 μg protein).

crystallised from green plants by SHIN, TAGAWA AND ARNON[8]. The plant enzyme catalyses the reversible reduction of ferredoxin (E_0' at pH 7 = —0.42 V) when NADPH is the hydrogen donor, whereas the Azotobacter particles (P6) utilize NADH only.

It is of interest that a non-haem iron protein from Azotobacter is also reduced by NADH in these particles. The observation that this reaction was inhibited at high

concentrations of non-haem iron and that this effect was removed when adequate NADH was generated suggests that the iron protein acts as an electron carrier in the coupled nitrate reductase system. By using electron spin resonance technique, NICHOLAS *et al.*[8] have shown that a non-haem iron protein in Azotobacter particles was markedly reduced by dithionite. These particles also catalyse H_2 evolution and N_2 fixation provided an ATP-generating system and dithionite are added[9,10].

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Oxidative phosphorylation in the metabolism of sarcosine and formaldehyde

It had been shown previously that the *N*-methyl groups of both sarcosine and dimethylglycine are metabolized to "active formaldehyde" in phosphate-washed mitochondria[1–3]. In these preparations, the "active formaldehyde" does not undergo further oxidation but is converted either to ordinary formaldehyde or to the β-carbon of serine. Subsequent studies, described below, have demonstrated that the metabolism of sarcosine in sucrose-washed mitochondria results in oxidative phosphorylation and also that the oxidation of the methyl group under these conditions proceeds beyond the level of "active formaldehyde" to formate and carbon dioxide.

With mitochondria washed 3 times with 0.25 M sucrose and then incubated with 0.01 M phosphate in sucrose, as described in Table I, the methyl group is oxidized beyond the level of formaldehyde to formate and carbon dioxide, and serine is a