Nitrogenase of *Klebsiella pneumoniae*: Interaction with Viologen Dyes as Measured by Acetylene Reduction

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Acetylene reduction by nitrogenase is a sensitive test for nitrogen fixation (see Postgate, 1971). Both acetylene reduction and nitrogen fixation require ATP and a suitable reductant, which may be $\text{Na}_2\text{S}_2\text{O}_4$ (Bulen *et al.*, 1965), ferredoxin (Mortenson, 1964) or Methyl Viologen (D'Eustachio & Hardy, 1964). Viologen dyes were reduced by hydrogen, pyruvate or KBH_4 for use with crude extracts of *Clostridium pasteurianum* (D'Eustachio & Hardy, 1964), or by NADH and NADH dehydrogenase for partially purified nitrogenase systems from *Azotobacter chroococcum* (Yates, 1971) and *Mycobacterium flavum* (Biggins & Postgate, 1971). These reports referred to relatively crude nitrogenase preparations and the specific activities recorded were very low; interaction of Viologen dyes with pure nitrogenase from *Klebsiella pneumoniae* was mentioned by Eady *et al.* (1972) but not studied in detail.

The present communication reports details of acetylene reduction by purified nitrogenase from *K. pneumoniae* with Benzyl or Methyl Viologen as electron donor, including an effect of Benzyl Viologen on alleviating inhibition of acetylene reduction by excess of the iron–molybdenum component of *K. pneumoniae* nitrogenase (Kp1) over the iron component (Kp2; nomenclature of Eady *et al.*, 1972).

Materials and methods

K. pneumoniae strain M5a1 was grown and harvested, and the nitrogenase was purified, handled and assayed for acetylene reduction with $\text{Na}_2\text{S}_2\text{O}_4$, as reported by Eady *et al.* (1972). Acetylene reduction with Viologen dyes was measured in serum bottles (7.5–8 ml) containing 40 μmol of 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid (HEPES) buffer, pH 7.8, 8 μmol of ATP, 20 μmol of MgCl_2 , 15 μmol of creatine phosphate and 0.05 μmol of Benzyl Viologen or 0.3 μmol of Methyl Viologen dye (or as indicated in Figs. 1 and 2) in a final volume of 1.5 ml. The bottle was flushed with H_2 and sealed under a stream of H_2 with a Suba-Seal (Griffin and George, Wembley, Middx., U.K.) closure. Then through this closure 0.5 ml of acetylene and 0.2 ml of the crude hydrogenase (prepared as described below) were injected and the vessel was shaken until the dye

was reduced (approx. 10 min at 30°C). It was important to have all of the dye reduced before the enzyme was added. The pressure in the bottle was then equalized to atmospheric pressure by piercing the Suba-Seal with a hypodermic needle, and reaction was started by injecting 0.2 mg of creatine kinase followed by purified nitrogenase. After the mixture had been shaken for 10 min at 30°C, reaction was stopped by injection of 0.1 ml of 40% (w/v) KOH and the ethylene produced was determined as described by Eady *et al.* (1972).

Fig. 1. Effect of Viologen concentrations on rate of acetylene reduction by nitrogenase from *K. pneumoniae* in the absence of alternative electron donors

Assays were conducted as described in the text with reduced Methyl Viologen (▽) or reduced Benzyl Viologen (○) as electron donor. Each contained 0.13 mg of Kp1 protein and 0.03 mg of Kp2 protein. In similar assays in which Viologen dye was replaced by 20 μmol of $\text{Na}_2\text{S}_2\text{O}_4$, 188 μmol of acetylene was produced/min per mg of protein.

Fig. 2. Effect of concentration of Benzyl Viologen on acetylene reduction by nitrogenase from *K. pneumoniae* at different ratios of Kp1 protein to Kp2 protein

For experimental details see the text. Curve A (∇), excess of Kp2 protein (0.13 mg of Kp1 protein: 0.1 mg of Kp2 protein); curve B ($\circ$), optimum Kp1/Kp2 ratio (0.13 mg of Kp1 protein: 0.03 mg of Kp2 protein); curve C ($\square$), excess of Kp1 protein (1.30 mg of Kp1 protein: 0.03 mg of Kp2 protein). In reactions with 13 mM- $\text{Na}_2\text{S}_2\text{O}_4$, 250 μM -reduced Methyl Viologen or 30 μM -reduced Benzyl Viologen as electron donor, the ratio of Kp1 protein to Kp2 protein used for curve C would give 25–30% of the activity with 200 μM -reduced Benzyl Viologen.

Hydrogenase was an unfractionated extract of acetone-dried *Desulfovibrio vulgaris* (strain Hildenbrough, N.C.I.B. 8303) that had been grown in a lactate-sulphate medium (Ware & Postgate, 1971). The preparation had no acetylene-reducing activity by itself.

Results and discussion

As shown in Fig. 1, optimum rates of acetylene reduction, at a Kp1/Kp2 molar ratio of 1:1, occurred over a narrow range of dye concentrations when reduced Benzyl Viologen was the electron donor, but

over a wide range of concentrations with reduced Methyl Viologen as electron donor. Tests with different concentrations of dye, and with Kp1/Kp2 molar ratios from 0.01 to 4.0, confirmed that the optimum molar ratio of nitrogenase components was 1:1 for acetylene reduction. With $\text{Na}_2\text{S}_2\text{O}_4$ or reduced Methyl Viologen as reductant, concentrations of Kp1 protein in excess of this equimolar ratio inhibited acetylene reduction, but with reduced Benzyl Viologen as reductant no such inhibition occurred. Excess of Kp2 protein was not inhibitory with any electron donor.

The optimum concentration of reduced Benzyl Viologen for acetylene reduction was independent of the concentration of Kp2 protein and of total protein, but was proportional to the concentration of Kp1 protein (Fig. 2). The optimum concentration of reduced Methyl Viologen for activity did not vary with concentrations of Kp1 protein, Kp2 protein or total protein.

With each electron donor at its optimum concentration the specific activity of the nitrogenase was similar with $\text{Na}_2\text{S}_2\text{O}_4$ or with reduced Methyl Viologen as electron donor, but with reduced Benzyl Viologen activity was lowered by about one-third.

These results show that reduced Methyl Viologen is as effective an electron donor to purified *Klebsiella* nitrogenase as is $\text{Na}_2\text{S}_2\text{O}_4$; reduced Benzyl Viologen is not so effective. Since the optimum concentration of reduced Benzyl Viologen is proportional to the concentration of Kp1 protein and reduced Benzyl Viologen can alleviate the inhibition produced by excess of Kp1 protein, it is probable that reduced Benzyl Viologen binds to specific sites on the uncomplexed Kp1 protein. This conclusion is supported by the observed inhibitory effect of excess of reduced Benzyl Viologen and the lower specific activities obtained with this reductant. Both of the latter observations suggest that the reduced Benzyl Viologen inhibits binding of Kp2 protein to Kp1 protein, thus preventing formation of the active nitrogenase complex.

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