EPR STUDIES OF NITROGENASE: ATP DEPENDENT OXIDATION OF FRACTION 1 PROTEIN BY CYANIDE

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1. Introduction

The enzyme system involved in the reduction of nitrogen to ammonia, nitrogenase, has been isolated from a number of aerobic bacteria, facultatively anaerobic bacteria and the strictly anaerobic bacterium *Clostridium pasteurianum* [1–3]. Nitrogenase will also catalyse the reduction of other compounds with triple bonds which therefore function as substrate analogues, for example acetylene and cyanide. The reduction of all of these substrates requires two proteins, ATP, and a low potential reductant such as sodium dithionite or reduced ferredoxin. It has not previously been shown which of the two proteins reacts with ATP and the substrate.

Both proteins are iron sulphur proteins containing non-haem iron and labile sulphur. One of the proteins (fraction 1 protein) which has a molecular weight of approximately 150,000 also contains molybdenum. The other protein (fraction 2 protein) is smaller (MW $\simeq$ 50,000) and does not contain molybdenum.

Iron sulphur proteins in the reduced state show characteristic EPR spectra, at low temperatures, centred at $g = 1.94$ [4]. Some, for example the iron sulphur protein from Azotobacter also has a spectrum in the oxidised form with a g value slightly greater than 2.00 [5]. Molybdenum also has an EPR spectrum in some redox states and it has been observed in molybdenum containing proteins [6]. Since nitrogenase proteins contain both molybdenum and iron sulphur groupings they might be expected to show characteristic EPR spectra which might assist in the investigation of the mechanism of action of nitrogenase. Fraction 2 protein from *Cl. pasteurianum* has been reported to have an EPR signal only in the oxidised state [7] while reduced Azotobacter nitrogenase has been reported to have signals at $g = 2.01$ and $g = 1.93$ [8]. We have investigated the EPR spectrum of fraction 1 protein of nitrogenase isolated from the green photosynthetic bacterium *Chloropseudomonas ethylicum*. We have obtained spectra of the oxidised and reduced forms of the enzyme and we have found that it can be oxidised by the substrate analogue cyanide in an ATP dependent reaction.

2. Methods

Chloropseudomonas ethylicum was grown as described previously [9]. Cell free extracts with nitrogenase activity were prepared using a Manton-Gaulin homogeniser. Nitrogenase proteins 1 and 2 were purified from these extracts by DEAE cellulose and the gel filtration chromatography essentially as described by other workers [2, 3]. Full details of the purification procedure and properties of the nitrogenase from this organism will be described elsewhere. All purification steps were carried out under anaerobic conditions. The final nitrogenase preparations were isolated in the reduced form in the presence of 0.1 mM $Na_2S_2O_4$.

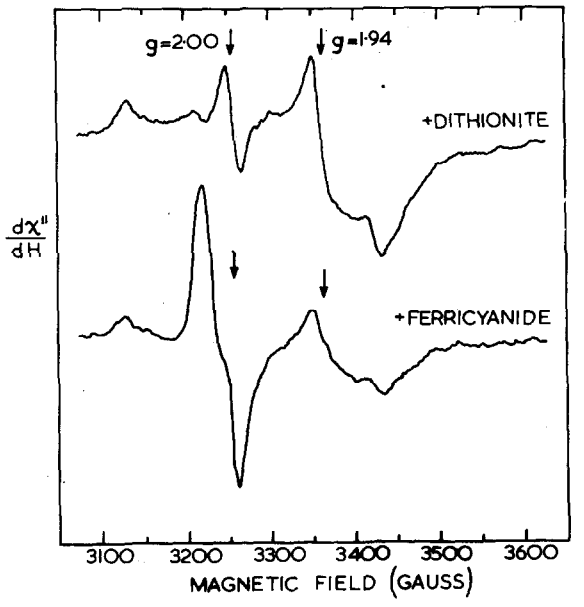

Fig. 1. EPR spectra of oxidised and reduced *C. ethylicum* nitrogenase fraction 1 protein. The spectra were recorded from 0.1 ml samples of a solution of fraction 1 protein (18 mg protein/ml), in 0.02 M tris-Cl buffer pH 7.5 containing 5 mM $MgCl_2$. The sample was reduced by adding 1 μmole of $Na_2S_2O_4$ or oxidised by adding 0.5 μmole of potassium ferricyanide. After incubation at 20° C for 1 min under argon the samples were frozen and stored in liquid nitrogen. The microwave power was 10 mW, the modulation amplitude 6.3 gauss, the microwave frequency 9.169 GHz and the scanning rate 1000 gauss/min. The temperature was 13° K.

Nitrogenase activity was assayed by measuring the reduction of acetylene [10]. The fraction 1 protein used in these experiments was entirely free from fraction 2 protein and showed no acetylene reducing activity in the absence of added fraction 2 protein.

Samples for EPR measurements were prepared anaerobically under argon and frozen in silica tubes of 3 mm internal diameter. EPR spectra were recorded using a Varian E4 spectrometer. During measurements the sample was cooled by blowing liquid helium past the sample tube which was held in an insert dewar of quartz inside the cavity of the spectrometer.

The temperature was measured by means of a gold + 0.03% iron versus chromel thermocouple, placed in the gas stream just below the sample tube. A small correction was applied to allow for the difference in temperature between the position of the thermocouple and the position of the sample.

3. Results and discussion

Fig. 1 shows EPR spectra obtained from fraction 1 protein. When reduced by sodium dithionite fraction 1 protein has the $g = 1.94$ signal characteristic of many iron sulphur proteins in the reduced form. On oxidation with excess potassium ferricyanide (or oxygen) the $g = 1.94$ signal is lost and is replaced by a signal in the region of $g = 2.04$. The reduced preparation also has a signal at $g = 2.00$ due to an unidentified free radical which increases in size on oxidation.

These spectra were obtained at 13° K; the nitrogenase signals are extremely temperature dependent and could not be observed at temperatures higher than 30° K.

The occurrence of easily recognisable EPR spectra in the oxidised and reduced forms of fraction 1 nitrogenase protein permits and investigation of the interaction of the protein with ATP and with possible substrates which might oxidise the protein. We have used cyanide as substrate as it can be used in solution more conveniently than nitrogen or acetylene. Fig. 2 shows the effects of adding either ATP, cyanide or ATP and cyanide together on the EPR spectrum of the reduced fraction 1 protein. The addition of either ATP or cyanide alone does not produce any marked effect on the EPR spectrum. However, on addition of ATP and cyanide together the $g = 1.94$ signal of the fraction 1 protein is replaced by the $g = 2.04$ signal. These changes are the same as those observed on oxidation of the fraction 1 protein by ferricyanide or oxygen. The oxidation by ATP and cyanide is observed only if the ATP and cyanide are in excess of the dithionite already present in the enzyme preparation. In similar experiments with fraction 2 protein preparations we have not observed any gross effect of ATP and cyanide on the EPR spectrum.

These experiments show that the fraction 1 protein is oxidised by cyanide, a substrate analogue, in an ATP dependent reaction. They indicate that fraction 1 protein is the site of substrate activation

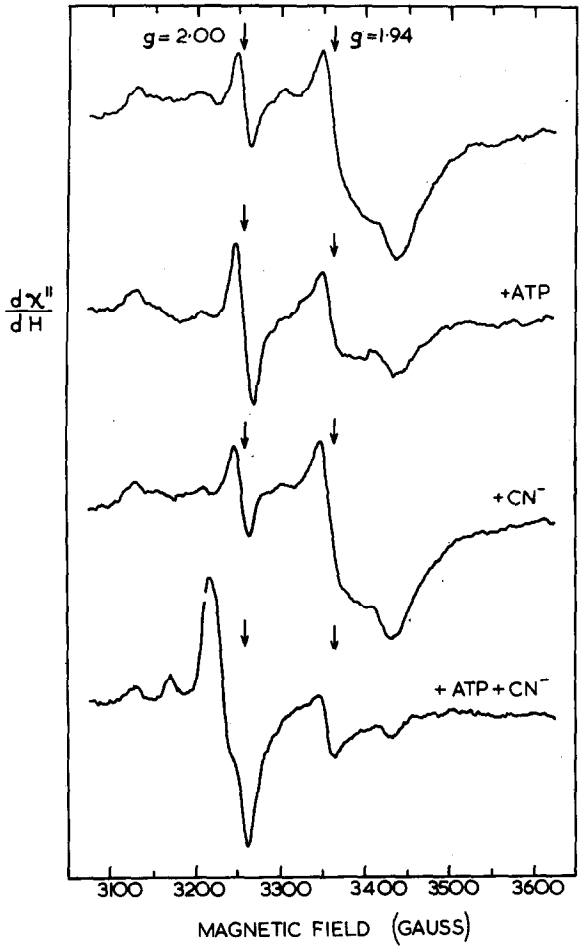

Fig. 2. The effect of ATP and cyanide on the EPR spectrum of reduced *C. ethylicum* nitrogenase fraction 1 protein. The reaction mixtures contained 0.1 ml of fraction 1 protein (18 mg protein/ml) in 0.02 M tris-Cl buffer pH 7.3 containing 5 mM $MgCl_2$. ATP 0.4 μmole (plus an ATP generating system of creatine phosphate 5 μmoles and creatine kinase 3 μg) and KCN 0.25 μmole were added as indicated. After incubation at 20° for 1 min under argon the samples were frozen and stored in liquid nitrogen. The spectra were recorded under the same conditions as in fig. 1.

and reduction by nitrogenase. They do not provide any explanation for the requirement for fraction 2 protein observed when nitrogenase activity is assayed by measurement of the products of the reaction or any evidence concerning the role of molybdenum in the mechanism of action of fraction 1 protein.

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