MEASUREMENT OF THE OXIDATION REDUCTION POTENTIAL OF THE EPR DETECTABLE ACTIVE CENTRE OF THE MOLYBDENUM IRON PROTEIN OF CHROMATIUM NITROGENASE

by

S.L. Albrecht* and M.C.W. Evans*

Department of Plant Sciences,

University of London King's College,

68 Half Moon Lane,

London SE24 9JF.

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Summary

The midpoint oxidation-reduction potential of the EPR detectable centre of the molybdenum iron protein of Chromatium nitrogenase has been measured. Two centres with identical EPR spectra but different midpoint potentials were detected. The measured midpoint potentials are (1) $Em_{7.5}$ = -60 mV and (2) $Em_{7.5}$ = -260 mV. The midpoint potentials were not affected by other components of the nitrogen fixing system.

INTRODUCTION

The enzyme complex, nitrogenase, which carries out the biological reduction of nitrogen has two component proteins, an iron protein and a molybdenum iron protein. The molybdenum iron protein which has been purified from *Azotobacter* sp (1,2) *Clostridium pasteurianum* (3) *Klebsiella pneumoniae* (4) and Chromatium (5) has a characteristic EPR spectrum with g values at 4.3, 3.7 and 2.01 in the reduced state. This signal is thought to be due to an iron complex with a net spin of $\frac{3}{2}$.

* Present address

Department of Botany and Microbiology,

University College,

Gower Street,

London. W.C.1.

If the protein is none destructively oxidised, this signal disappears. In the presence of the iron protein, ATP and excess reductant, the signal also disappears. It is not clear whether this disappearance results from the substrate dependent oxidation of the protein (6) or its reduction to a more reduced form (5,7,8). We have now measured the oxidation reduction potential of the EPR detectable centre of the molybdenum iron protein in an attempt to define the changes which occur on oxidation and reduction.

MATERIALS AND METHODS

Chromatium Str D was grown and nitrogenase prepared from it as described previously (5). Potential measurements were made on preparations at various stages of purification, from crude protamine sulphate precipitates to essentially pure molybdenum iron protein. Oxidation reduction potential titrations were done essentially by the procedure of Dutton (9) in an anaerobic vessel, continuously flushed with oxygen free nitrogen. The oxidation reduction potential was measured using a platinum electrode (Radiometer, Copenhagen Type P101) and a standard calomel electrode (Radiometer, Copenhagen Type K401). Samples were transferred to pre-gassed EPR tubes through a stainless steel transfer tube and frozen under a stream of nitrogen. The following compounds were used as mediators between the electrode and the nitrogenase: Methyl viologen (E_o' = -440 mV) 200 μM; benzyl viologen (E_o' = -311 mV) 200 μM, Phenosafranine (E_o' = -239 mV) 100 μM, 2-hydroxy 1,4,naphthoquinone (E_o' = -149 mV) 100 μM, methylene blue (E_o' = +11 mV) 100μM.

The potential of the reaction mixture was adjusted using either 0.1M potassium ferricyanide, saturated Louth's violet (thionine) (approximately 1.8 mg/ml) or 1% sodium dithionite in 0.1M Tris-Cl buffer pH 9.0. Titrations were carried out by oxidising reduced samples and then reducing them back to the original state. EPR spectra were obtained using a Varian E4 spectrometer. Samples were cooled to 10-15°K by a stream of helium gas passing through a quartz dewar inside the EPR cavity. The temperature was measured by a thermocouple placed upstream of the sample.

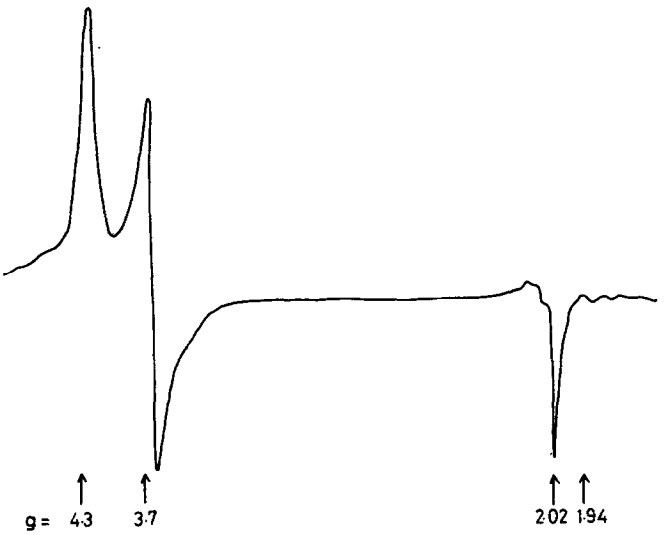

Figure 1
EPR spectrum of Chromatium nitrogenase molybdenum iron protein. The spectrum was recorded at 18°K with the following instrument settings. Frequency 9.170 GHz; power 10 mW, modulation amplitude 109, scan rate 100G/min., gain 2.5×10^2. Protein concentration 40 mg/ml.

RESULTS AND DISCUSSION

Figure I shows the EPR spectrum of a purified sample of Chromatium nitrogenase molybdenum iron protein used in these experiments. This shows the characteristic spectrum with signals at $g = 4.3$, 3.7 and 2.01. In partially purified preparations containing both the molybdenum iron protein and the iron protein a large signal centred at $g = 1.94$ due to the iron protein, and possibly contaminating iron sulphur proteins, is also present. In the potential titrations measurements of signal size were based on the height of the $g = 3.7$ signal. This part of the signal is characteristic of nitrogenase, and is the most satisfactory part of the signal to measure. The $g = 2.01$ component is partially concealed by the large free radical signal of the reduced mediators, while the $g = 4.3$ signal is close to the nonspecific iron signal which may be present in partially purified preparations and also the signals at $g = 4.4$ which appears on destructive oxidation of the protein.

Figure 2 shows the oxidation reduction potential titration of the

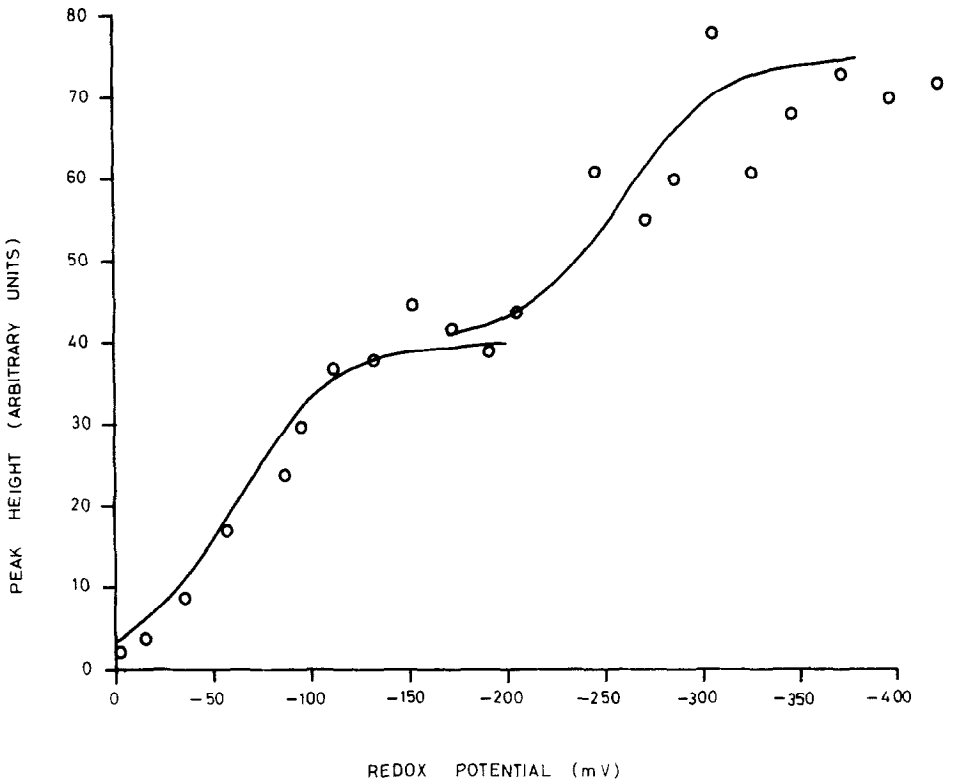

Figure 2

Oxidation reduction potential titration of the g = 3.7 signal of Chromatium nitrogenase. The titration was carried out as described in the text. Spectra were recorded as in Fig. 1 with suitable gain settings and the peak height of the signal taken as a measure of signal size. O experimental points. The lines drawn are the theoretical curves for a one electron accepting centre with the observed midpoint potentials.

purified molybdenum iron protein. The titration clearly shows that two centres with different midpoint potentials are present in the protein. One centre has a $Em_{pH\ 7.5} = -60$ mV and the other $Em_{pH\ 7.5} = -260$ mV. (A variation of ± 10 mV was observed in the midpoint potential measurement in the course of ten titrations). As far as we can discover these two centres have identical EPR spectra. The lines drawn on Figure 2 are theoretical curves for single electron accepting centres with these midpoint potentials. The fit to these lines is good showing that the centres are reduced by a single electron. The titration shown is a reductive titration

of Lauths Violet oxidised material, similar results were obtained in oxidative titrations with Lauths Violet. If ferricyanide was used as oxidant the centre with $Em_{7.5}$ = -260 mV was usually destroyed and only the centre at $Em_{7.5}$ = -60 mV could be reversibly oxidised and reduced. After titration with Lauths Violet and dithionite the nitrogenase retained its activity, ferricyanide titration resulted in loss of most of the activity. The two centres appear to make an equal contribution to the EPR signal, although some variation was observed between experiments.

Zumft and Mortenson (10) have reported changes in the midpoint potential of the iron protein on addition of magnesium and ATP. We have measured the midpoint potentials of the molybdenum iron protein in the presence of magnesium, ATP and an ATP generating system, and also in the presence of the iron protein and of the iron protein ATP and magnesium. We have not detected any significant change in the midpoint potentials of the molybdenum iron protein under these conditions. It is therefore unlikely that ATP interacts directly with these centres of the molybdenum iron protein. We have not detected any decrease in signal size during titrations of mixtures of the proteins in the presence of ATP and magnesium, although a decrease is seen in the presence of a large excess of dithionite. It seems likely that further reduction of the protein must occur at potentials lower than those which can be measured by this technique (about -440 mV at pH 7.5). It is however possible that under the conditions of the titration the rate of reduction of the molybdenum iron protein by the iron protein and ATP is slower than the substrate reduction rate. Under these conditions the molybdenum iron protein would remain largely in the dithionite reduced state and no change would be observed.

Eady *et al* (7) have reported that two electrons per mole are required for the complete reduction of the EPR signal. Together with our results this indicates that each molecule contains two one electron accepting centres. The similarity of the EPR spectra of the two centres suggest

that they are very similar, the difference in midpoint potential resulting from the different environment of the centres. The large differences between the midpoint potentials, and the relatively high potential of one centre, make it seem unlikely that they function together at a substrate reduction site. Our results therefore support the suggestion that further reduction of these centres is necessary before substrate reduction can occur.

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