Electrochemical titration of the $S = 3/2$ and $S = 1/2$ states of the iron protein of nitrogenase

T. Vance Morgan, Roger C. Prince and Leonard E. Mortenson*

EXXON Research and Engineering, Annandale, NJ 08801, USA

Received 21 July 1986

The iron protein of nitrogenase delivers electrons and ATP to the iron-molybdenum protein, which in turn reduces dinitrogen to ammonia. The iron protein contains a single four-iron, four-sulfur prosthetic group, detectable by ESR spectroscopy in its reduced form. Until recently, spin quantitations suggested that only a portion of the reduced iron protein was being detected by ESR, but recent work in several laboratories has shown that there is a spin = 3/2 signal near $g = 5$ in addition to the well characterized spin = 1/2 signal near $g = 1.94$. In this paper we characterize the redox properties of both states in the presence and absence of ATP and ADP, and find that the new spin state has identical redox properties to those previously determined for the spin = 1/2 state.

(A. vinelandii, C. pasteurianum) *Nitrogenase* *Iron protein* *Redox titration* *EPR*

1. INTRODUCTION

The iron protein component of nitrogenase is involved in bringing electrons and Mg-ATP to the iron-molybdenum protein for the overall reduction of dinitrogen to ammonia [1,2]. The iron protein is a two-subunit protein, containing a single 4Fe-4S center which is believed to be held between the two subunits by cysteine residues [3]. Electron transfer from the reduced iron protein to the iron-molybdenum protein occurs only when the reaction is coupled with the hydrolysis of Mg-ATP molecules [4]. The iron protein binds 2 Mg-ATP molecules [5,6] and the hydrolysis product Mg-ADP is an inhibitor of the nitrogenase reaction [7].

Binding of Mg-ATP to the iron protein is accompanied by several changes in the properties of the protein. The ESR spectrum ($g = 1.94$) shifts from a rhombic line shape to an axial form still near $g = 1.94$, while the measured redox potential drops from about −300 mV to about −400 mV [8,9]. Binding of Mg-ATP further increases the oxygen lability of iron protein and increases reactivity of the iron toward chelating agents [10].

The ESR spectrum integrated in the $g = 2.0$ region of the spectrum has consistently given intensities < 1; on average about 0.3 spins/4 Fe [11–15]. Recent work in several laboratories has addressed this problem [16–18] and the essence of the new findings is that, at least in the case of *Azotobacter vinelandii* iron protein, there are two spin states. One of these is the previously known $S = 1/2$ system at $g = 1.94$; the other an $S = 3/2$, $g = 5$ signal. Taken together these signals give about 1 spin/4 Fe. Further, in 50% ethylene glycol the intensity of the $S = 1/2$ form increases and essentially all the $S = 3/2$ form disappears, while the converse is found in 0.4 M urea [16].

The physiological importance of the distinct forms of the protein is unknown, and no information is available about their relationship to the redox behavior of the iron protein. Furthermore, there is some dispute as to whether the redox

* Present address: Dept of Biochemistry, University of Georgia, Athens, GA 30602, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

behavior of *A. vinelandii* and *Clostridium pasteurianum* iron protein is the same and as to whether the redox processes show Nernstian $n = 1$ behavior [19].

We have reinvestigated the midpoint potentials of both *C. pasteurianum* and *A. vinelandii* iron protein with and without Mg-ATP and Mg-ADP and in addition we have investigated the redox properties of the $S = 3/2$ form of the *A. vinelandii* iron protein, and the effect of ethylene glycol on this behavior.

2. MATERIALS AND METHODS

C. pasteurianum (strain W5) and *A. vinelandii* (strain OP) nitrogenase were prepared from frozen cell paste as described [20,21]. All operations were carried out under an argon atmosphere, either on a gas manifold or in a Vacuum Atmospheres glove box. All buffers were Ar-equilibrated and contained 1 mM sodium dithionite except for the redox titrations. Nitrogenase acetylene reduction assays were carried out at 30°C in 8 ml septum sealed vials as in [22]. Samples were assayed on a Varian 6000 gas chromatograph at 30°C using a Poropak R column (Waters Associates). All samples in these experiments were of high activity (*C. pasteurianum* 1700–2000 nmol ethylene formed $\cdot \min^{-1} \cdot mg^{-1}$ and *A. vinelandii* 2200 nmol ethylene formed $\cdot \min^{-1} \cdot mg^{-1}$).

ESR spectra were taken on a Varian E 109 spectrometer equipped with an Oxford Instruments continuous flow cryostat. Instrumental conditions are listed in the figure legends.

ATP, ADP and ethylene glycol were obtained from Sigma; ethylene glycol was distilled before use. All other chemicals were reagent grade or of the highest purity available.

Redox potentiometry followed the method outlined by Dutton [23], and used a platinum mesh measuring electrode and a saturated calomel reference electrode. Titrations typically used 2–12 mg/ml iron protein in 100 mM Tricine, pH 8.0, with 40 μM benzyl and methyl viologens, indigodisulfonate and 2-hydroxy-1,4-naphtho- and anthraquinones as mediators. Tricine was used as buffer to minimize pH changes during freezing of the ESR samples [24]. Sodium dithionite was used as the reductant and thionine (E_{m8} 40 mV [25]) as the oxidant because of concerns that ferricyanide might irreversibly oxidize the iron-sulfur center [26]. The redox cell was in a glove box (O_2 < 2 ppm) and samples were taken after 10 min equilibration at each potential. Samples were placed in ESR tubes that were capped inside the glove box, withdrawn and immediately frozen in liquid nitrogen-chilled iso-octane. Titrations typically involved 10 samples (i.e. a total volume of 3 ml) and started with fully reduced protein. The titration took about 2–3 h and after this time about 85% of the signal was recovered upon full reduction. All potentials are reported with respect to the standard hydrogen electrode.

Protein determinations were by the method of Lowry et al. [27].

3. RESULTS AND DISCUSSION

Fig.1 shows ESR data gathered from the *A. vinelandii* iron protein. Samples with and without Mg-ATP and ethylene glycol are shown. The

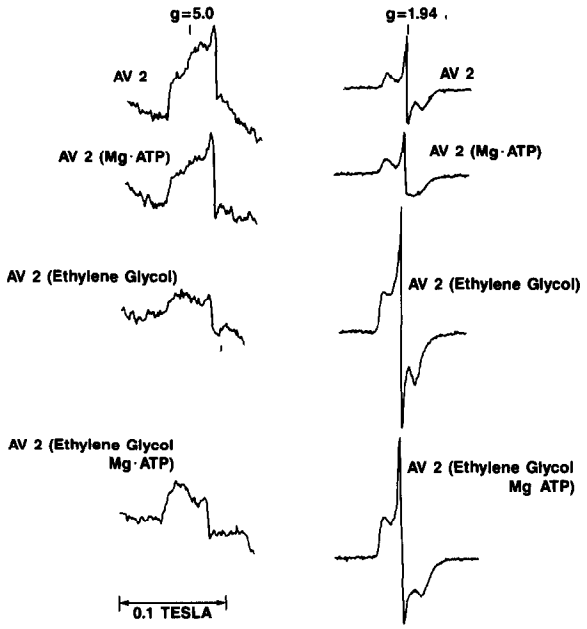

Fig.1. ESR spectra of *A. vinelandii* iron protein (AV2). Iron protein (9.5 mg/ml) in 50 mM Tricine, 1 mM dithionite, pH 8.0, with 2.5 mM ATP, 3.8 mM $MgCl_2$ and 50% (v/v) ethylene glycol as indicated. ESR conditions: for the $g = 2$ region, 0.2 mW applied power, 1 mT modulation, 10 K; for the $g = 5$ region, 20 mW applied power, and gain increased 6.3-fold. Microwave frequency 9.23 GHz.

previously reported line shape change with Mg-ATP and the observed increase in $g = 2$ intensity with glycol are clearly seen. The point of interest lies in the spectrum with both Mg-ATP and glycol present simultaneously. The conversion to axial line shape is incomplete. This suggests that glycol may interfere with Mg-ATP binding or that conformational changes of the protein in glycol are not the same as in buffer alone. The remaining figures will show how the 'native' and glycol-induced ESR states relate to the electrochemical redox potential with and without Mg-ATP or ADP.

Titration of *C. pasteurianum* and *A. vinelandii* iron protein was followed by measuring loss of intensity of the $g = 1.94$ peak of the ESR spectrum. The titration of *C. pasteurianum* iron protein is displayed in fig.2. An E_{m8} of -266 mV is found for the form without Mg-ATP and the E_m is not changed by ATP alone. The data are displayed fitted to an $n = 1$ Nernstian curve (upper panel, fig.2). The fit is excellent and there is no indication of an alternate process. The lower panel of fig.2 shows the effect of Mg-ATP or Mg-ADP. The data are again fitted to an $n = 1$ process with good agreement, but the E_{m8} has dropped to -415 mV. Both Mg-ATP and Mg-ADP are equally effective in causing the negative shift. The upper panel of fig.3 shows a comparison of the behavior of the $S = 3/2$ and $S = 1/2$ forms of the *A. vinelandii* iron protein taken in a single titration experiment. Both fit an $n = 1$ process with an E_m of -280 mV and there is no detectable difference in redox behavior of either the $S = 3/2$ or $S = 1/2$ form. Just as with the *C. pasteurianum* protein, the addition of Mg-ATP causes a shift in the E_m to -430 mV, as shown in the lower panel of fig.3.

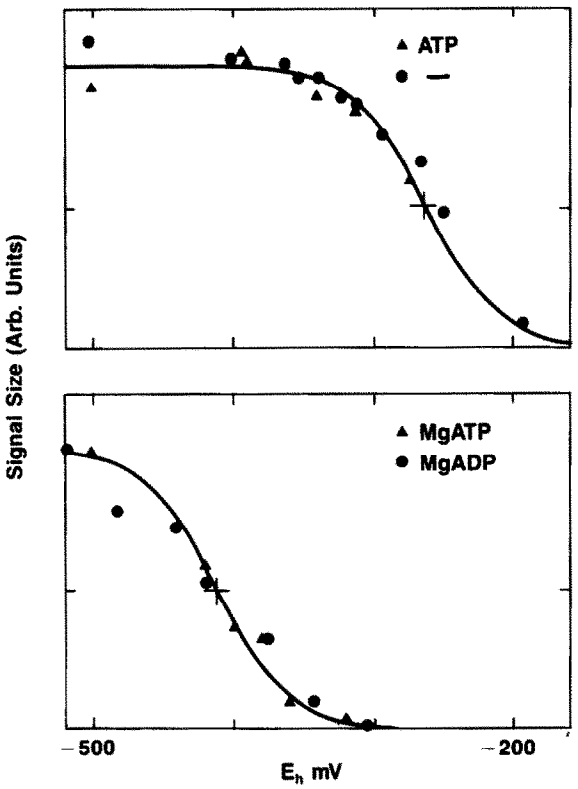

Fig.2. Redox titrations of *C. pasteurianum* iron protein. Iron protein (2.1 mg/ml) in 50 mM Tricine, pH 8.0, with 0.78 mM ATP, 0.70 mM ADP and 1.4 mM $MgCl_2$ as indicated. ESR conditions: 2 mW applied power, 1 mT modulation, 13 K, 9.23 GHz.

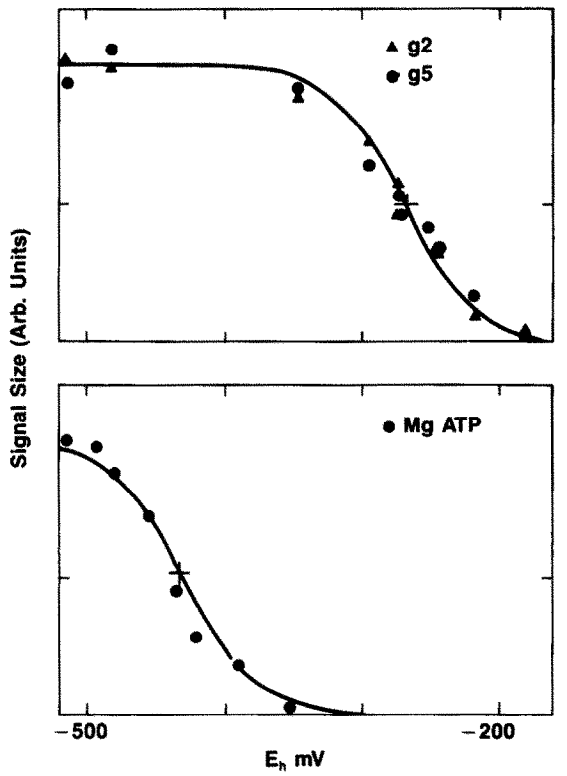

Fig.3. Redox titrations of *A. vinelandii* iron protein. Upper panel: iron protein (12 mg/ml), ESR conditions as in fig.1. Lower panel: iron protein (9.0 mg/ml) with 1 mM ATP, 1.8 mM $MgCl_2$, ESR conditions as in fig.1.

Fig.4 compares the ESR behavior of the $g = 5$ ($S = 3/2$) and $g = 2$ ($S = 1/2$) forms of the *A. vinelandii* iron protein in the presence and absence of ATP. The straight line behavior of the relationships shows that both signals follow the same redox process. One can conclude, therefore, that no distinction of physiological role can be made using a redox midpoint difference. While the $g = 5$ signal appears in *C. pasteurianum* iron protein, it was somewhat broader and more difficult to quantitate for titration purposes. Nevertheless, our experiments show qualitatively similar behavior to that shown in fig.4.

Figs 2–4 thus show that the iron proteins from *C. pasteurianum* and *A. vinelandii* exhibit almost identical redox behavior in both the presence and the absence of Mg-ATP, and at least for the protein of *A. vinelandii*, there is no difference in the redox behavior of the $S = 3/2$ and $S = 1/2$ spin states of the iron-sulfur cluster. We see no evidence for anything other than the expected Nernstian $n = 1$ behavior, and indeed it is hard to envisage how an ESR-detectable species, which must have an unpaired electron to be detectable, could titrate as a Nernstian $n = 2$ couple [19].

The addition of 50% ethylene glycol to the iron protein shifted the E_m to -200 mV. A possible explanation of this positive shift of the midpoint may be the partial loss of water from the protein or the iron-sulfur center. Proposals have been made [28–30] that the redox potentials of iron-sulfur

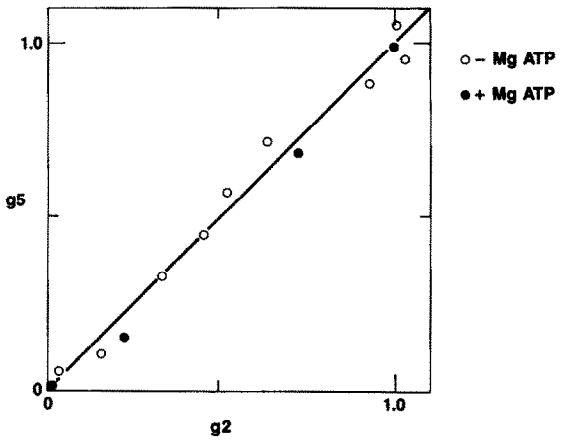

Fig.4. Comparison of the $g = 2$ and $g = 5$ signals of the *A. vinelandii* iron protein during redox titrations such as those of fig.3.

centers are influenced by both the hydrophobic nature of the protein and the formation of stable hydrogen bonds between NH groups of the peptide chain and sulfur atoms of the Fe_4S_4 centers or the terminal sulfurs of cysteine residues. Further, Odell and Geary [31] showed that the E_m shifted 170–200 mV more positive for a synthetic Fe_4S_4 center in aqueous solution when bound into bovine serum albumin or insulin as compared to potentials in water. If the $S = 3/2$ center is an 'extended-distorted' cube as Lindahl et al. [16] suggested, partial stabilization of the center may be due to a particular hydrogen bonding which is subsequently altered in the more hydrophobic environment of 50% ethylene glycol to allow the stabilization of the $S = 1/2$ form. The $S = 1/2$ form is further perturbed by either an interaction with ethylene glycol or a slightly altered hydrogen bonding permitted in the more hydrophobic environment of 50% glycol.

Titration of the *A. vinelandii* iron protein in the presence of 50% ethylene glycol and Mg-ATP (8-fold molar excess) gave irreversible results. The ESR signal of the iron protein was completely lost at -350 mV and could not be regenerated on addition of excess dithionite reductant. The further alteration of iron protein structure on binding of Mg-ATP in the presence of glycol possibly exposes the iron-sulfur center in such a way that as the center is oxidized, hydrolysis of the center occurs or glycol may become a ligand and denature the center.

Electrochemical titration of the iron protein from two different microorganisms has shown that the process is a 1-electron process for both cases, and that the newly discovered $S = 3/2$ form of *A. vinelandii* iron protein is indistinguishable from the $S = 1/2$ form in its redox midpoint and fit to an $n = 1$ Nernst curve. This suggests that the two spin states can rapidly interconvert, but whether either form is the exclusive form for activity remains unclear.

REFERENCES

[1] Orme-Johnson, W.H. (1985) Annu. Rev. Biophys. Chem. 14, 419–459.

[2] Burgess, B.K. (1984) in: Advances in Nitrogen Fixation Research (Veeger, C. and Newton, W.E. eds) pp.103–113, Nijhoff/Junk, The Hague, Netherlands.

[3] Hausinger, R.P. and Howard, J.B. (1983) J. Biol. Chem. 258, 13486–13491.
[4] Thorneley, R.N.F., Lowe, D.J., Eady, R.R. and Miller, R.W. (1979) Biochem. Soc. Trans. 7, 633–636.
[5] Hageman, R.V., Orme-Johnson, W.H. and Burris, R.H. (1980) Biochemistry 19, 2333–2342.
[6] Cordweiner, J., Haaker, H. and Veeger, C. (1983) Eur. J. Biochem. 132, 47–54.
[7] Kennedy, I.R., Morris, J.A. and Mortenson, L.E. (1968) Biochim. Biophys. Acta 153, 777–788.
[8] Zumft, W.G., Mortenson, L.E. and Palmer, G. (1974) Eur. J. Biochem. 46, 525–535.
[9] Lowe, D.J., Smith, B.E. and Eady, R.R. (1980) in: Recent Advances in Nitrogen Fixation (Rao, S. ed.) pp.34–87, Oxford and IBH, New Delhi.
[10] Mortenson, L.E. and Thorneley, R.N.F. (1979) Annu. Rev. Biochem. 48, 387–418.
[11] Palmer, G., Multani, J.S., Cretney, W.C., Zumft, W.G. and Mortenson, L.E. (1972) Arch. Biochem. Biophys. 153, 325–332.
[12] Zumft, W.G., Palmer, G. and Mortenson, L.E. (1973) Biochim. Biophys. Acta 292, 413–421.
[13] Orme-Johnson, W.E., Davis, L.C., Henzyl, M.T., Averill, B.A., Orme-Johnson, N.R., Munck, E. and Zimmerman, R. (1977) in: Recent Developments in Nitrogen Fixation (Newton, W. and Postgate, J.R. eds) pp.133–178, Academic Press, New York.
[14] Anderson, G.L. and Howard, J.B. (1984) Biochemistry 23, 2118–2122.
[15] Haaker, H., Braaksma, A., Cordweiner, J., Klugquist, J., Wassink, H., Eady, R.R. and Veeger, C. (1984) in: Advances in Nitrogen Fixation Research (Veeger, C. and Newton, W.E. eds) pp.123–131, Nijhoff/Junk, The Hague, Netherlands.
[16] Lindahl, P.A., Day, E.P., Kent, T.A., Orme-Johnson, W.H. and Munck, E. (1985) J. Biol. Chem. 260, 1160–1173.
[17] Hagen, W.R., Eady, R.R., Dunham, W.R. and Haaker, H. (1985) FEBS Lett. 189, 250–254.
[18] Watt, G.D. and McDonald, J.W. (1985) Biochemistry 24, 7226–7231.
[19] Braaksma, A., Haaker, H., Grande, H.J. and Veeger, C. (1982) Eur. J. Biochem. 121, 483–491.
[20] Mortenson, L.E. (1972) Methods Enzymol. 29, 446–456.
[21] Burgess, B.K., Jacobs, D.J. and Steifel, E.I. (1980) Biochim. Biophys. Acta 614, 196–209.
[22] Mortenson, L.E., Walker, M.N. and Walker, G.A. (1976) Proc. 1st Int. Symp. Nitrogen Fixation (Newton, W.E. and Nyman, C.J. eds) pp.117–149, Wash. State Univ. Press.
[23] Dutton, P.L. (1978) Methods Enzymol. 54, 411–435.
[24] Williams-Smith, D.L., Bray, R.C., Barber, M.J., Tsopanakis, A.D. and Vincent, S.P. (1977) Biochem. J. 167, 593–600.
[25] Clark, W.M. (1972) Oxidation Reduction Potentials of Organic Systems, Robert E. Krieger, Huntington, NY.
[26] Morgan, T.V., Stephens, P.J., Devlin, F., Stout, C.D., Melis, K.A. and Burgess, B.K. (1984) Proc. Natl. Acad. Sci. USA 81, 1931–1935.
[27] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[28] Lode, E.T., Murray, C.L. and Rabinowitz, J.C. (1976) J. Biol. Chem. 251, 1683–1688.
[29] Rabinowitz, J.C. and Sweeney, W.V. (1980) Annu. Rev. Biochem. 49, 139–161.
[30] Adman, E., Watenpaugh, K.D. and Jensen, L.H. (1975) Proc. Natl. Acad. Sci. USA 72, 4854–4858.
[31] Odell, B. and Geary, P.J. (1984) J. Chem. Soc. Dalton Trans. 29, 29–36.