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Electron-Paramagnetic-Resonance Studies on Nitrogenase of *Klebsiella pneumoniae*

EVIDENCE FOR ACETYLENE- AND ETHYLENE-NITROGENASE TRANSIENT COMPLEXES

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Klebsiella pneumoniae nitrogenase exhibited four new electron-paramagnetic-resonance signals during turnover at 10°C, pH 7.4, which were assigned to intermediates present in low concentrations in the steady state. ^{57}Fe-substituted Mo–Fe protein showed that they arose from Fe–S clusters in the Mo–Fe protein of nitrogenase. The new signals are designated: I_c, g values at 4.67, 3.37 and approx. 2.0; VI, g values at 2.125, 2.000 and 2.000; VII, g values at 5.7 and 5.4; VIII, g values at 2.092, 1.974 and 1.933. The sharp axial signal VI arises from a Fe_4S_4 cluster at the −1 oxidation level. This signal was only detected in the presence of ethylene and provides the first evidence of an enzyme–product complex for nitrogenase. [^{13}C]Acetylene and [^{13}C]ethylene provided no evidence for direct binding of this substrate and product to the Fe–S clusters giving rise to these signals. The dependence of signal intensities on acetylene concentration indicated two types of binding site, with apparent dissociation constants $K < 16\,\mu$M and $K \sim 13$ mM. A single binding site for ethylene ($K = 1.5$ mM) was detected. A scheme is proposed for the mechanism of reduction of acetylene to ethylene and inhibition of this reaction by CO.

Nitrogenase of *Klebsiella pneumoniae* consists of two proteins, Kp1, mol.wt. 218000, and Kp2, mol.wt. 67000 (Eady *et al.*, 1972). Kp1 protein is tetrameric and contains two each of two types of subunit of mol.wts. 50000 and 60000 (Kennedy *et al.*, 1976). It contains 33±3 Fe atoms and 2 Mo atoms per tetramer (Smith *et al.*, 1976*b*). Kp2 protein has two identical subunits and contains four Fe atoms and four acid-labile sulphide ions per dimer. The analogous protein from *Clostridium pasteurianum*, Cp2, has been shown to contain a single Fe_4S_4 cluster in which alternate corners of a cube are occupied by Fe^{2+} or Fe^{3+} and S^{2-} ions (Gillum *et al.*, 1977). The position of the Fe_4S_4 cluster with respect to the two peptide chains is not known.

E.p.r. (Smith *et al.*, 1972, 1973), Mössbauer (Smith & Lang, 1974) and stopped-flow (Thorneley, 1975; Thorneley & Cornish-Bowden, 1977) spectroscopy showed that Kp2 protein donates electrons to Kp1 protein in a MgATP-dependent reaction that is rapid ($k = 2.0\times10^2\,s^{-1}$ at 23°C) relative to the catalytic-centre activity for the enzyme (approx. $2\,s^{-1}$). On reduction, Kp1 protein loses its e.p.r. signal at $g = 4.3$, 3.6 and 2.0. Signals of this type have been assigned to the spin $S = \frac{1}{2}$ ground state of a spin $S = \frac{3}{2}$ system (Palmer *et al.*, 1972; Smith *et al.*, 1973; Münck *et al.*, 1975). Corresponding changes in Mössbauer parameters indicated that two Fe_4S_4 clusters (45% of the iron) become reduced, and that the remaining Fe atoms are distributed in at least two other environments. The steady-state concentration of intermediates in which these Fe atoms have been oxidized or reduced must be low, since no changes in their Mössbauer parameters were detected in Kp1 or Av1 proteins on entering the steady state (Smith & Lang, 1974; Münck *et al.*, 1975).

A second type of redox-active Fe_4S_4 centre in Cp1 protein has been detected by e.p.r. spectroscopy under conditions where electron flux to acetylene or N_2 was inhibited by CO but H_2 evolution maintained (Orme-Johnson & Davis, 1977; Davis *et al.*, 1978). At low partial pressures of CO a net charge of −3 was assigned to the centre with $g = 2.08$, 1.97 and 1.93. At higher partial pressures of CO the centre became oxidized to the −1 oxidation level, with $g = 2.17$ and 2.05. Since no evidence for direct binding of ^{13}CO to the centre was obtained, it was suggested that at low partial pressures of CO the electron flux between the new centre and the N_2-reduction site was inhibited, causing the Fe_4S_4 centre to become more reduced relative to the steady state in the absence of CO. At higher partial pressures, binding of CO to a second class of site or sites interrupts electron flow to the centre, causing it to become oxidized.

Abbreviations used: the nitrogenase components of the various organisms are denoted by a capital letter indicating the genus and a lower-case letter the species and the number 1 or 2 denotes which of the protein components is referred to. The number 1 indicates the Mo–Fe-containing protein and the number 2 the Fe-containing protein. Kp, *Klebsiella pneumoniae*; Cp, *Clostridium pasteurianum*; Av, *Azotobacter vinelandii*; Ac, *Azotobacter chroococcum*.

Yates & Lowe (1976) observed a fourth type of e.p.r. signal with Ac and Kp nitrogenase, with $g = 2.140$, 2.001 and 1.976, for Ac1 protein in the steady state. Since the intensity of this signal was only approx. 0.02 electron per molecule of active Ac nitrogenase complex, this centre must be associated with an intermediate present only in low concentration under turnover conditions. This signal was not detected in the presence of acetylene, cyanide or azide, and it was suggested that this centre may be involved in H_2 evolution.

The distribution of electrons between the various redox centres at equilibrium will be determined by the relative redox potentials of the centres. Substrate, product or inhibitor binding, or changes in pH or temperature, may all cause a redistribution of electrons within the nitrogenase complex by altering the relative redox potentials of the Fe–S and Mo centres, resulting in new or altered e.p.r. signals. Kinetic factors may also affect the electron distribution. Thorneley & Eady (1977) observed differential kinetic effects for reduction of N_2, acetylene and protons by lowering the assay temperature from 30 to 10°C and by using high molar ratios of Kp1 to Kp2 protein. Hence it was decided to use a recently developed e.p.r. system with improved data-handling characteristics (Bray *et al.*, 1978), giving better signal resolution, to monitor nitrogenase under these conditions in the hope that the altered relative rates of substrate reduction would be reflected in a different distribution of electrons over the various Fe–S centres of Kp1 protein in the steady state. An e.p.r. investigation under the conditions of Thorneley & Eady (1977) is complicated by the lag phase for acetylene reduction and a complementary burst phase for H_2 evolution. The present paper is concerned only with e.p.r. signals and their intensities in the steady state after the lag phase is complete (times greater than approx. 15min) and in particular with the effects of acetylene and its reduction product ethylene on these signals.

Materials and Methods

The component proteins of *Klebsiella pneumoniae* nitrogenase were purified as described by Eady *et al.* (1972), with an additional final DEAE-cellulose chromatography step (Smith *et al.*, 1976*b*). The specific activities of the component Kp1 and Kp2 proteins when assayed under the conditions of Eady *et al.* (1972) at 30°C were 1470 and 1200nmol of ethylene produced/min per mg of protein respectively. Kp1 protein substituted with ^{57}Fe was prepared as described by Smith & Lang (1974). Protein solutions contained 25mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH7.4, and 10mM-$MgCl_2$. Stock solutions of proteins and other reagents were diluted to give a final assay volume of 0.56ml containing, except where stated, 6μM-Kp2 protein, 19μM-Kp1 protein, 18mM-phosphocreatine, 20mM-$MgCl_2$, 27mM-$Na_2S_2O_4$, 18mM-ATP and 50μg of creatine kinase. The creatine kinase and phosphocreatine were included to prevent the accumulation of inhibitory concentrations of MgADP. Doubling the concentration of creatine kinase had no effect on the H_2-evolution activity at 10°C, which under these conditions remained constant for at least 45min. All biochemicals and creatine kinase (EC 2.7.3.2) were purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K., and salts were from BDH, Poole, Dorset, U.K. Pure Ar obtained from Air Products Ltd., New Malden, Surrey, U.K., was used as the inert gas in all experiments. Ethylene (Air Products) and acetylene generated from calcium carbide were scrubbed with $Na_2S_2O_4$ solution before use. G.l.c. failed to detect any CO in any of the above gases, indicating a concentration of less than 0.1p.p.m. (by vol.).

Assays were carried out in serum bottles, volume 7ml, fitted with rubber Suba-Seal closures, thermostatically maintained at 10°C in a shaker bath oscillating at 100 strokes/min. Assays were started by syringe addition of ATP to assay bottles containing all other reagents and proteins under an atmosphere of Ar with various proportions of ethylene, acetylene or CO. At the appropriate time, the total liquid content of the assay bottle (0.56ml) was removed with a precooled Ar-flushed disposable plastic syringe fitted with a 30cm 20-gauge stainless-steel needle and then syringed into an e.p.r. tube that had been thermostatically maintained at 10°C on an all-glass vacuum line in the absence of O_2 [<0.5p.p.m. (by vol.)]. After being filled, the e.p.r. tube was plunged into isopentane at −140°C to freeze the sample. The complete transfer process took approx. 15s. During this time, 0.5ml of 30% (w/v) trichloroacetic acid was injected into the essentially empty assay bottle to quench the activity of any residual protein. The gases in the assay bottle were then analysed by g.l.c. We stress the details of the assay, since, even with a 15s transfer time, the enzyme will have undergone a number of turnovers, and, since temperature is an important parameter in these studies, every attempt has been made to keep the assay mixture at constant temperature at every stage in the transfer process. Another necessary precaution is not to fill the transfer syringe too quickly. A partial vacuum over the solution during filling would have perturbed the equilibria involving the gaseous substrates, products and inhibitors with the protein solution. Undoubtedly our failure to overcome these problems completely contributed considerably to the scatter in the data presented in the Figures. It was not possible to carry out the assay in an e.p.r. tube, thus avoiding transfer problems, because

adequate gas equilibration with the assay mixture requires a large surface area relative to the depth of the solution and a shaking motion.

Assays under Ar or Ar plus ethylene were run for 30min before transfer to an e.p.r. tube. All assays involving acetylene were run for 28min under Ar or Ar plus ethylene, and then acetylene was added for the final 2min before transfer. This procedure was used to minimize conversion of acetylene into ethylene during the assay. The concentrations of acetylene and ethylene dissolved in the protein solutions were calculated from the volume of gas added and experimentally determined absorption coefficients. Liquid- and gas-phase samples were injected with a gas-tight syringe on to a Poropak R column and the amounts of ethylene and acetylene determined. The absorption coefficient was then calculated at 10°C for solutions identical with those used in the assays.

E.p.r. techniques were as described by Lowe & Bray (1978). The various signals were measured from difference spectra at three different temperatures (29K, 18K and 12K). Spectra run at 29K were used to estimate signal V (see nomenclature below) from the height of its g_3 component (only the g_2 component of signal VIII can interfere, and this signal was not observable at this temperature). After the appropriate amount of signal V has been subtracted from the 29K spectrum, signal VI (see the Results section) can be estimated from its $g_\perp$ component. At 18K signal II (from Kp2 protein) can be measured from its high-field features and then subtracted, leaving the g_3 component of signal VIII as a good measure of this signal; under some conditions other signals overlapped the g_1 component of signal VIII, so that this feature was not found to be a good measure of its intensity. Signal IV is also measured from its $g_\perp$ component at 18K. Finally all signals were subtracted from the 18K spectrum to ensure that no other species were present. Spectra at 12K enable signals VII, I_a, I_b and I_c to be measured from their g_1 and g_2 components.

Computer simulations of e.p.r. spectra were carried out with the BASIC program designed by Lowe (1978).

E.p.r. nomenclature

The e.p.r. signals shown by nitrogenase have previously been referred to by the conditions under which they may be observed, e.g. 'low'- and 'high'-CO signal. Since in the present paper we have shown that at least one of these signals can be obtained in the absence of CO and because of the increasing numbers of signals observed, we have used a numerical system of nomenclature (Table 1). We stress that different signals may be given by the same centre in different states of the enzyme. Signals that are clearly given by the same centre have been assigned the same number, with letters to distinguish different enzyme forms.

Calculation of binding constants

Apparent binding constants (K) associated with changes in the intensity of e.p.r. signals were calculated by using a least-squares fitting procedure to either the equation $s = Sv/(K+v)$ or $s = SK/(K+v)$ according to whether the signal intensity increased or decreased respectively as more acetylene or ethylene was added. s is the measured signal height, v the quantity of gas added and S is the maximum signal height. Points were weighted in proportion to their density on the v axis. The ranges quoted for the values of K in Table 2 are the values within which the sum-of-the-squares error increased by less than 25% above the value at the minimum.

Results

The e.p.r. spectra described below were those observed in the functioning enzyme in samples frozen after a reaction time of 30min, a time chosen to be well outside the lag period for acetylene reduction at 10°C (Thorneley & Eady, 1977).

E.p.r. signals under Ar

Four distinct e.p.r.-active species can be identified in the steady state under Ar. Signals I_a and I_b are the high- and low-pH forms given by the spin $S = \frac{3}{2}$ centre of Kp1 protein and have approx. 10% of the intensity of controls to which no ATP had been added. Signal V, with g values of 2.139, 2.001 and 1.977, is similar to that reported by Yates & Lowe (1976) for Ac nitrogenase in the steady state at 30°C. Under our steady-state conditions this signal gives an integrated intensity of 0.021 electron per Kp1 molecule.

Signal VIII, with g values of 2.092, 1.974 and 1.933 (Figs. 1*a* and 1*b*), has an integrated intensity of 0.036 electron per Kp1 molecule and is very similar to that reported for Av nitrogenase in the steady state when substrate reduction (other than protons) was inhibited by low partial pressures of CO (Burris & Orme-Johnson, 1976; Davis *et al.*, 1978). It is not, however, identical with this signal, since, in experiments in which we exposed Kp nitrogenase to stoicheiometric concentrations of CO, we observed a signal with g values at 2.073, 1.969 and 1.927 (Fig. 1*d*). In addition, signal VIII, unlike the signal induced by low partial pressures of CO, is not observable at 30K. We have designated the true low-CO signal as signal III and the signal observed under Ar alone as signal VIII. When the Kp1 protein was substituted with ^{57}Fe (nuclear spin $\frac{1}{2}$) a 3mT increase in linewidth was observed (Fig. 1*c*).

Signal VII, which has features at $g = 5.7$ and 5.4

Table 1. *E.p.r. signals associated with the nitrogenase proteins of K. pneumoniae isolated in the presence of $Na_2S_2O_4$ and in mixtures of component proteins in the steady state during turnover*

Designation	g values	$g_{av.}$	Protein	Comments	Reference
I_a	4.32, 3.63, 2.009	—	Kp1	Protein as isolated, low-pH form, decreased intensity during turnover	Smith *et al.* (1973)
I_b	4.27, 3.73, 2.018	—	Kp1	Protein as isolated, high-pH form, decreased intensity during turnover	Smith *et al.* (1973)
I_c	4.67, 3.37, $\approx$2.0	—	Kp1	Only observed during turnover under acetylene, intensity enhanced by ethylene	The present work
II_a	2.053, 1.942, 1.865	1.953	Kp2	Protein as isolated	Smith *et al.* (1973)
II_b	2.036, 1.929 (g_m)	1.965	Kp2	MgATP-bound form	Smith *et al.* (1973)
II_c	g values poorly defined, but intermediate between IIa and IIb	—	Kp2	MgADP-bound form	D. J. Lowe, B. E. Smith & M. G. Yates (unpublished work)
III	2.073, 1.969, 1.927	1.990	Kp1	CO bound tightly, only observed during turnover	The present work
IV	2.17, 2.06, 2.06	2.10	Kp1	CO bound weakly, only observed during turnover	The present work
V	2.139, 2.001, 1.977	2.039	Kp1	Only observed during turnover; may be associated with H_2 evolution	The present work
VI	2.125, 2.000, 2.000	2.042	Kp1	Ethylene-bound form only observed during turnover, decreased by acetylene	The present work
VII	5.7, 5.4	—	Kp1	Only observed during turnover	The present work
VIII	2.092, 1.974, 1.933	2.000	Kp1	Only observed during turnover	The present work

(Fig. 2*a*), has not been previously reported for nitrogenase. When the Kp1 protein was substituted with ^{57}Fe, the linewidth of signal VII increased from 2.8mT to 3.4mT as shown in Fig. 2(*b*). No other features of this signal were observed at magnetic-field strengths less than 0.5T. Spin lattice relaxation causes signal VII to broaden above 20K. Between 12K and 20K there is less than a 10% change in intensity after correction for the temperature term in the Boltzmann distribution; thus signal VII does not arise from a state that is a member of a manifold with energy separation corresponding to about 10–20K, i.e. it does not arise from the excited state of the spin $S = \frac{3}{2}$ system.

Signals I_a, I_b and V have previously been shown to arise from the Mo–Fe protein (Eady & Smith, 1978; Orme-Johnson & Davis, 1977). The intensity of all the e.p.r. signals was unchanged by the addition of H_2 at 28kPa (0.28atm) or 0.7kPa (0.007atm) to the assays.

E.p.r. signals under Ar plus ethylene

A new e.p.r. species, signal VI, appeared in the presence of ethylene (Fig. 3*a*). This axial signal has $g_{\parallel} = 2.125$ and g_m (the g value of the cross-over of the first derivative spectrum) = 2.007; computer simulations gave $g_{\perp} = 2.000$. The use of ^{57}Fe-substituted Kp1 protein caused the peak-to-peak linewidth of the $g_{\perp}$ feature to increase from 2.0 to 3.0mT (Fig. 3*b*).

The intensity of signal VI as a function of ethylene partial pressure is shown in Fig. 4 together with the effect of ethylene on the intensities of signals I_a, I_b, I_c, V, VII and VIII. These latter signals have also been observed under Ar in the absence of ethylene. The apparent binding constants for ethylene, calculated from the changes in intensities of signals V, VI and VII (Fig. 4), are given in Table 2. The three values lie within experimental error of each other with an average value of 1.5mM, consistent with all three signals monitoring ethylene binding at a single class of site(s).

E.p.r. signals under Ar plus acetylene

The intensities of signals I_a, I_b, V, VII and VIII varied with the partial pressure of acetylene (Fig. 5). These signals were also observed in the absence of acetylene. In addition, two new signals were

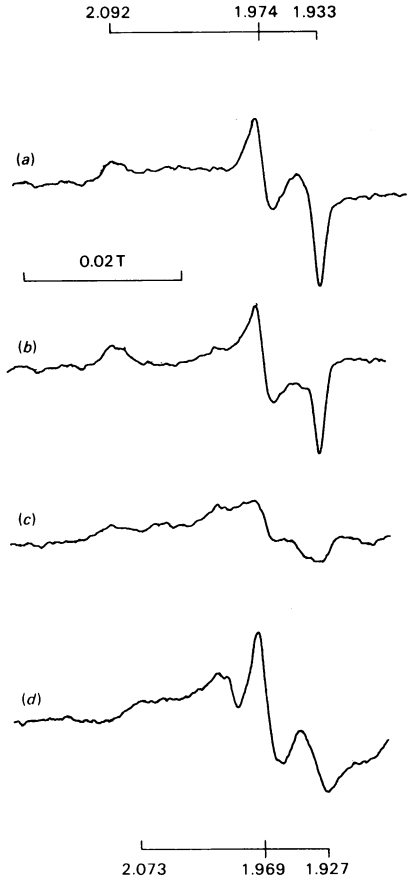


Fig. 1. *E.p.r. signals VIII and III of [^{56}Fe]Kp1 protein of nitrogenase together with signal VIII of [^{57}Fe]Kp1 protein of nitrogenase*

Fig. 1(*a*) shows the e.p.r. spectrum of Kp nitrogenase containing [^{56}Fe]Kp1 protein frozen 30min after initiation of catalytic activity at 10°C by addition of ATP. Incubation was under Ar for 28min, followed by a 2min incubation with 8.3mM-acetylene. Trace (*b*) is the result of subtracting the spectrum of Kp2 protein from trace (*a*) so that all the high-field features corresponding to Kp2 protein have been removed, and illustrates a pure signal VIII. Trace (*c*) is the spectrum of a sample similar to that of (*a*), except that ^{57}Fe-substituted Kp1 protein (sp. activity 612nmol of ethylene produced/min per mg of protein) was used, showing a 3mT line-broadening of the g_3 component. Spectrum (*d*) was given by a sample frozen 4min after initiation of catalytic activity at 25°C under Ar plus 0.08% CO by addition of ATP. It illustrates signal III contaminated with the spectrum of Kp2 protein. E.p.r. was carried out at 18K by using field modulations of 0.5mT for (*a*), (*b*) and (*c*), and 1mT for (*d*) at 100kHz. The microwave power and frequency were 20mW and 9.162GHz respectively. The stick diagram at the top shows signal VIII (cf. traces *a*, *b* and *c*) and that at the bottom signal III (cf. trace *d*).

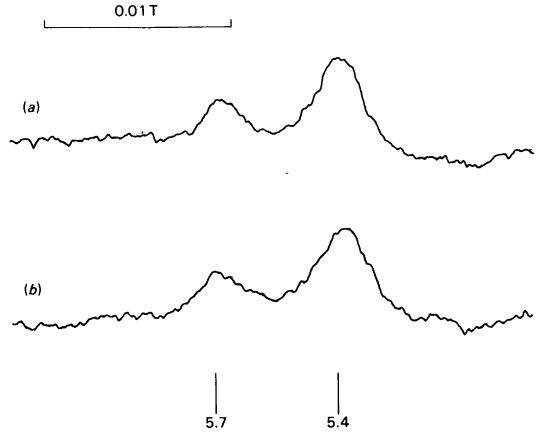


Fig. 2. *E.p.r. signal VII of nitrogenase with [^{56}Fe]- and [^{57}Fe]-Kp1 protein*

Spectrum (*a*) is given by a sample of Kp nitrogenase frozen 20min after initiation of catalytic activity at 10°C by addition of ATP under an Ar atmosphere. The Kp1 protein contained ^{56}Fe. Spectrum (*b*) is given by a similarly prepared sample with [^{57}Fe]Kp1 protein and shows a 0.6mT line-broadening. The reaction time was 30min under an Ar atmosphere containing ethylene (3mM in solution) at 10°C. E.p.r. was carried out at 12K by using 1.6mT field modulation at 100kHz. The microwave power and frequency were 150mW and 9.160GHz respectively. The *g* values of 5.7 and 5.4 are assigned as shown.

observed under acetylene. The first new signal, I_c (Fig. 6), has *g* values at 4.67, 3.37 and approx. 2.0, and was not observed at 30K. Since its temperature variation is the same as that of signal I, which has been shown to be associated with Fe, and Mo(V) and Mo(III), e.p.r. spectra are observable at much higher temperatures (Lowe *et al.*, 1972; Jarrett, 1957), signal I_c is presumably not an Mo centre and arises from an increased rhombic distortion of the spin $S = \frac{3}{2}$ centre. The second signal (Fig. 7*a*) was only observed when the partial pressure of acetylene lay between 4Pa (4×10^{-5}atm) and 40kPa (0.4atm) (Fig. 5). This signal was indistinguishable from signal IV (Fig. 7*b*), which has previously only been observed in the presence of high partial pressures of CO (Orme-Johnson & Davis, 1977). However, since this signal is weak and details are obscured by other signals we prefer only to tentatively identify it as signal IV. The apparent binding constants for acetylene calculated from the changes in signal intensities shown in Fig. 5 are given in Table 2.

The values clearly indicate two classes of acetylene-binding sites. Since [Kp1 protein] = 19μM, signals IV, V and VIII monitor tight stoicheiometric binding to a site with $K<20\mu$M, and signals IV, VII and VIII a looser binding site with K 1–13mM. Values of K

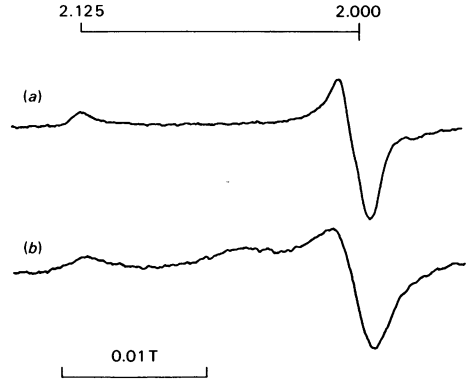


Fig. 3. *E.p.r. signal VI from* [^{56}*Fe*]- *and* [^{57}*Fe*]-*Kp*1 *protein of nitrogenase*

Fig. 3(*a*) shows the e.p.r. spectrum of [^{56}Fe]Kp1 nitrogenase frozen after 30min reaction time with 2.1mM-ethylene in solution (under Ar). Trace (*b*) is of a similar sample with [^{57}Fe]Kp1 protein (sp. activity 612nmol of ethylene produced/min per mg of protein) and shows a 1.0mT line-broadening. E.p.r. was performed at 29K by using a field modulation of 0.5mT at 100kHz. The microwave power was 20mW at a frequency of 9.157GHz. The stick diagram shows the g values of signal VI.

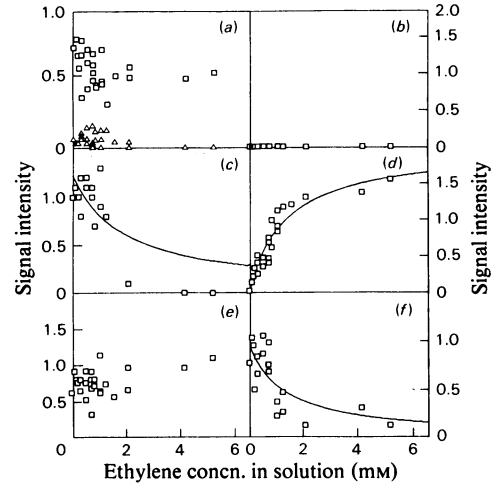


Fig. 4. *Variation of e.p.r. signal intensities during an ethylene titration*

The signals plotted are: (*a*), signal I; (*b*), signal IV; (*c*), signal V; (*d*), signal VI; (*e*), signal VII; (*f*), signal VIII. In plot (*a*) the squares correspond to the sum of the intensities of signals I_a and I_b and the triangles to signal I_c. For each signal a value of 1 on the ordinate scale corresponds to: (*a*), 22% of the intensity of the signal obtained in the absence of ATP; (*b*), arbitrary units; (*c*), 0.021 electron/molecule of Kp1 protein; (*d*), 0.034 electron/molecule of Kp1 protein; (*e*), arbitrary units; (*f*), 0.01 electron/molecule of Kp1 protein. The curves in (*c*), (*d*) and (*f*) are for the best-fit simple-binding equations with the K values as defined in Table 2. Samples were exposed to the appropriate concentrations of ethylene for 30min at 10°C before freezing in isopentane.

corresponding to individual signals have been included in Table 2, since they were used in the construction of the lines in Fig. 5. There is some evidence for the existence of multiple loose-binding sites, since signal VIII increases and then decreases in intensity in the high range of acetylene concentrations.

Signal VI was not observed in the presence of acetylene and Ar (see the next section).

Effect of ethylene on acetylene binding

The effect of a fixed partial pressure of ethylene, corresponding to approx. 50% saturation of the ethylene-binding site, on the binding of acetylene was investigated. The behaviour of the intensities of signals I_a, I_b, V and VII as a function of acetylene concentration was essentially unchanged by the presence of ethylene (compare Fig. 8 with Fig. 5). The marginal increase in signal I_c, apparent with increasing acetylene partial pressures, was enhanced by ethylene (Fig. 8). Signal VI, which was only observed in the presence of ethylene, decreased in intensity at acetylene partial pressures greater than 0.4kPa (0.004atm), and at partial pressures above 10kPa (0.1atm) this signal was undetectable, consistent with competitive binding of acetylene and ethylene.

The maximum intensity of signal IV and the range of acetylene concentrations over which it remains unchanged were both decreased in the presence of ethylene.

Signal VIII initially decreased in intensity as the partial pressure of acetylene increased, as was also observed in the absence of ethylene. At higher partial pressures of acetylene this signal reappeared, increased to a maximum and then decreased in intensity. The apparent binding constants for acetylene for these complex changes are tabulated in Table 2. As was observed with acetylene alone, the data indicate two classes of binding sites.

Time course of substrate reduction

Under the conditions of the e.p.r. experiments with high protein concentrations at 10°C, the rate of H_2 evolution under Ar was constant for at least 45min, with a specific activity of 43nmol of H_2 evolved/min per mg of Kp2 protein. In the presence of ethylene at 30kPa (0.3atm) the rate of H_2 evolution

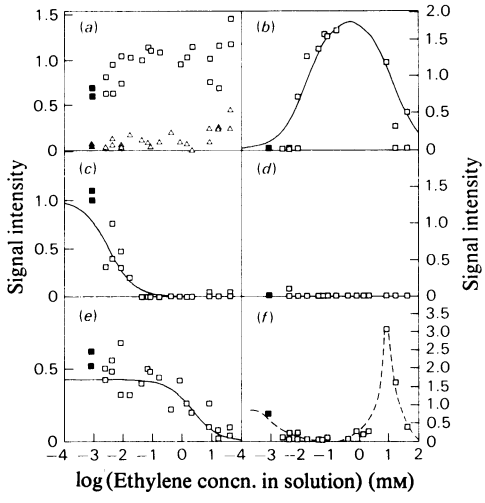


Fig. 5. *Variation of e.p.r. signal intensities during an acetylene titration*

The signals and ordinate scales are as in Fig. 4. The solid points correspond to the intensities for zero acetylene concentrations ($-\infty$ on a logarithmic scale). The solid curves are for the best-fit simple-binding equations with the K value as in Table 2, and the broken curve in (f) is hand-drawn, since the complexity of the effects preclude a full analysis at this stage. Samples were exposed to the appropriate concentrations of acetylene for 2min after a pre-incubation under Ar for 28min at 10°C.

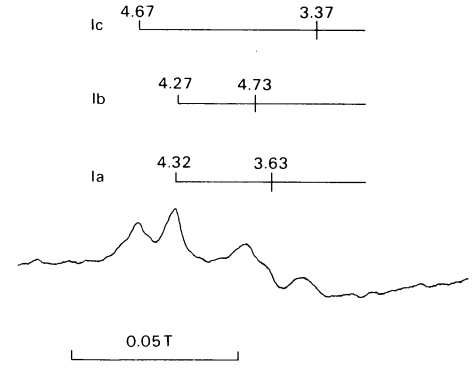


Fig. 6. *E.p.r. signals I_a, I_b and I_c of Kp protein of nitrogenase*

The sample was frozen after exposure for 1min to 2.5mM-acetylene plus 0.7mM-ethylene after pre-incubation for 29min with 0.7mM-ethylene plus Ar to 1atm at 10°C. The stick diagrams show the positions of the g_1 and g_2 features of signals I_a, I_b and I_c. E.p.r. was carried out at 12K by using a field modulation of 1.6mT at 100kHz. The microwave power was 150mW at a frequency of 9.200GHz.

increased to 76nmol/min per mg of Kp2 protein. The rate of ethylene formation in the presence of acetylene at a partial pressure of 13kPa (0.13atm) accelerated during a lag phase to become constant after 10min, with a specific activity of 63nmol of ethylene/min per mg of Kp2 protein with concomitant evolution of 13.4nmol of H_2/min per mg of Kp2 protein.

Discussion

The observation of four new e.p.r. signals that are only present under turnover conditions provides important parameters for mechanistic and structural studies on nitrogenase. In the present paper we have described these signals and used some of them to monitor the binding of acetylene and its reduction product ethylene to the Mo–Fe protein.

Signal VI, which was only observed in the presence of ethylene and whose intensity was decreased by acetylene, has been assigned to a complex with ethylene bound to Kp1 protein, present in low concentrations in the steady state. The apparent dissociation constants for ethylene ($K = 1.5$mM) and acetylene ($K < 16\mu$M), calculated by using signals VI, VIII or V, demonstrate weaker

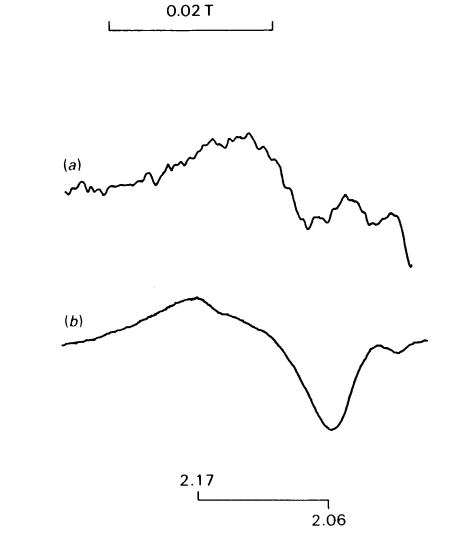


Fig. 7. *E.p.r. signal IV of Kp nitrogenase*

The sample giving trace (a) had been exposed to 0.067mM-acetylene for 2min after preincubation for 28min under Ar at 10°C. That giving trace (b) had been exposed to 0.29atm of CO (plus Ar to 1atm) for 30min at 10°C. E.p.r. was carried out at 18K by using 1mT field modulation at 100kHz. The microwave power was 20mW at a frequency of 9.161GHz. The gain in (a) is 10 times that in (b).

Table 2. *Apparent binding constants for acetylene and ethylene to K. pneumoniae nitrogenase calculated from e.p.r. signal intensities*

The values of K were calculated from the data in Figs. 4, 5 and 8 as described in the Materials and Methods section.

Titration	Class of binding site	Signals	K (mM)	Error range (mM)
Ethylene	Weak	VI	1.3	1.0–1.5
	Weak	VIII	1.3	0.7–2.3
	Weak	V	1.9	1.0–4.0
Acetylene	Tight	VIII	$<10^{-3}$	
	Tight	V	3.2×10^{-3}	2.6×10^{-3}–3.7×10^{-3}
	Tight	IV	16×10^{-3}	10×10^{-3}–20×10^{-3}
	Weak	VIII	1.3	0.6–2.1
	Weak	VII	2.3	1.1–4.2
	Weak	IV	13	6–18
Acetylene in the presence of ethylene	Tight	VIII	$<5\times10^{-3}$	
	Tight	V	6.2×10^{-3}	3.8×10^{-3}–9.2×10^{-3}
	Tight	VI	8×10^{-3}	1.5×10^{-3}–14×10^{-3}
	Tight	IV	60×10^{-3}	40×10^{-3}–85×10^{-3}
	Weak	IV	0.58	0.41–0.77
	Weak	VIII	2.5	1.6–3.5
	Weak	VII	3.0	1.9–4.4
	Weak	I_c	5.0	2.5–87
	Weak	VIII	16	9–22

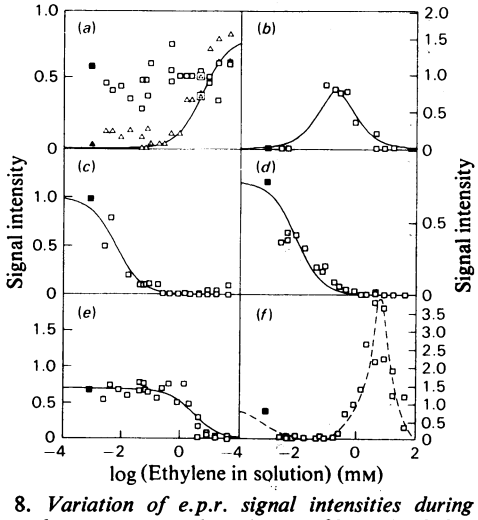


Fig. 8. *Variation of e.p.r. signal intensities during an acetylene titration in the presence of* 0.7 mM-*ethylene*

The signals, scales, points and curves are as in Fig. 5. Samples were exposed to the appropriate concentrations of acetylene plus ethylene for 2 min after preincubation at 10°C for 28 min under 0.7 mM-ethylene alone (plus Ar to 1 atm).

binding of the product ethylene than the substrate acetylene to the same site on the protein. Signal VI is not observed in the steady state when ethylene is being produced from acetylene. Therefore the release of bound ethylene, formed from acetylene, must be rapid relative to the rate-limiting step in acetylene reduction for the enzyme–ethylene complex not to accumulate to detectable amounts.

Signal VI, with all g values greater than 2.000 and $g_{av.} = 2.042$, presumably arises from an oxidized Fe_4S_4 cluster [centre X, species (*d*) and (*h*) in Scheme 2] at the −1 oxidation level (Orme-Johnson & Sands, 1973). Since signal VI was broadened by using ^{57}Fe-substituted Kp1 protein (Fig. 3*b*) centre X must be in Kp1 and not Kp2 protein. In the ethylene titration (Fig. 4), as the intensity of signal VI increases, the intensities of signals V and VIII decrease. The assignment of signals V and VIII to particular clusters and oxidation levels is difficult. Signal V, although its $g_{av.}$ is over 2.000, has one of its g values below 2.000. Therefore this signal cannot unequivocally be assigned to a classical oxidized high-potential iron protein type cluster (from *Chromatium vinosum*). Signal V may arise from a Fe_4S_4 cluster of unusual geometry at the −3 or −1 oxidation levels (see Schemes 1*a* and 1*b* respectively).

Since signal VIII has $g_{av.} = 2.000$, we cannot assign an oxidation level or structure to the centre from which this signal originates. However, the sum of the maximum integrations of signals V (2.1%) and VIII (3.6%) does approximately equal the integration of signal VI (5.1%), which could indicate that these signals arise from interconverting centres. A rhombic e.p.r. signal with $g_{av.} = 2.01$ with similar temperature and saturation behaviour to signals V and VIII, but with a greater linewidth, arises from a reduced Fe_2S_2 cluster in xanthine oxidase (EC 1.2.3.2) (Lowe *et al.*, 1972). Smith & Lang (1974) from Mössbauer data suggested that Fe_2S_2 clusters may be present in Kp1 protein.

[^{13}C]Ethylene caused no detectable (<0.03mT) broadening of signal VI. Thus there is no evidence for direct binding of ethylene to centre X. This is similar to the effect of ^{13}CO on Cp nitrogenase, which also failed to broaden the e.p.r. signal of an Fe_4S_4 cluster (centre Y in Scheme 1*a*). Increasing concentrations of CO caused centre Y to become first reduced, then oxidized. Davis *et al.* (1978) interpreted this as CO binding to two sites on either side of centre Y on the electron-transport chain but not directly to the cluster, and causing the electron flux into and out of centre Y to change. The question arises as to how good a probe of CO or ethylene binding to an Fe_4S_4 cluster the ^{13}C-line-broadening technique is. Erbes *et al.* (1975) successfully demonstrated ^{13}CO binding to an Fe_4S_4 cluster in Cp hydrogenase by observing a 0.2mT broadening, and on this basis we consider that if ethylene were bound directly to an e.p.r.-active Fe_4S_4 cluster we would have observed a corresponding degree of broadening.

We have assigned signals IV and VI to different Fe_4S_4 centres, Y and X respectively, since, although they both have $g_{av.} > 2.000$, they have very different linewidths and g values. In addition, as signal VI increases in intensity in the titration under ethylene alone (Fig. 4), signal IV is absent. If signals IV and VI arise from the same cluster one might expect signal IV to decrease as signal VI increased. Indistinguishable e.p.r. parameters for signal IV were obtained with high partial pressures of CO and in the absence of CO at intermediate concentrations of acetylene. This supports the suggestion of Davis *et al.* (1978) that CO does not bind directly to cluster Y.

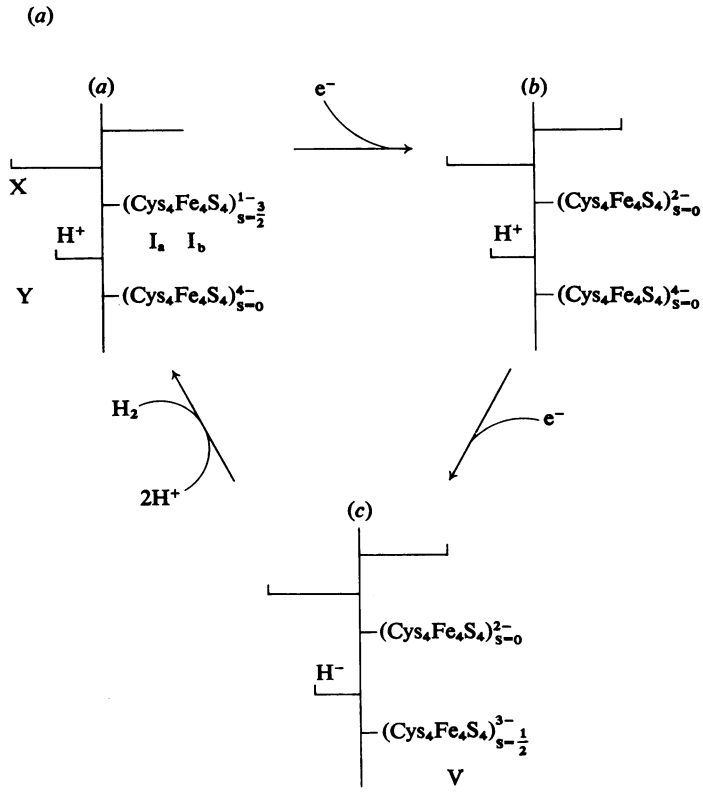
We propose the following schemes as working hypotheses for the mechanism of H_2 evolution (Schemes 1*a* and 1*b*) and acetylene reduction and CO inhibition (Scheme 2) consistent with e.p.r. data presented in this paper, those of Davis *et al.* (1978) on CO binding to Cp nitrogenase and the Mössbauer data of Smith & Lang (1974) and Münck *et al.* (1975). Mössbauer studies of ^{57}Fe-substituted proteins have shown that only 45% of the Fe atoms in isolated Kp1 and Av1 proteins are associated with the centre giving rise to the e.p.r. signals at $g = 4.3$, 3.7 and 2.01. On entering the steady state these Fe atoms become non-magnetic and have been assigned to Fe_4S_4 clusters at the −2 (e.p.r.-silent) oxidation level (Smith & Lang, 1974). In Schemes 1 and 2 the small amount of unresolved magnetic species observed in the steady state (approx. 5%) has been assigned on the basis of our e.p.r. data to Fe_4S_4 clusters at the −1 and −3 oxidation levels. Both Schemes are based on the minimum number of Fe_4S_4 clusters required to explain these data. The nature of the binding sites for protons, acetylene, ethylene and CO is not specified, although binding to e.p.r.-silent Mo or Fe atoms would be reasonable considering their chemical properties. A common binding site for acetylene and ethylene has been assumed, since ethylene is the product of acetylene reduction. However, we do not wish to imply that the oxidation state of the metal atoms or conformation of this site is the same for the enzyme–substrate (acetylene) and enzyme–product (ethylene) complexes. A separate high-affinity CO-binding site has been assumed, since CO is a non-competitive inhibitor of acetylene reduction (Hwang *et al.*, 1973). We have omitted a number of equilibria in both schemes for the sake of clarity and do not imply, for instance, that CO only binds when acetylene is also bound at the other site, as in species (i) (Scheme 2). For the moment we wish to consider the simplest models that can accommodate the main observations in this paper and those of Davis *et al.* (1978).

We have found it necessary to propose two schemes for H_2 evolution (Schemes 1*a* and 1*b*) because of the uncertainty in the assignment of an oxidation level to the cluster giving rise to signal V; Scheme 1(*a*) utilizes centre Y at the −3 oxidation level and Scheme 1(*b*) centre X at the −1 oxidation level.

Scheme 1(*a*)

Reduction of species (*a*) *to* (*b*). Species (*a*) is Kp1 protein as isolated in the presence of dithionite ion. Centre X gives rise to signals I_a and I_b. The protolytic equilibria that determine the relative intensities of signals I_a and I_b (Smith *et al.*, 1973) have not been included in Scheme 1(*a*) for the sake of clarity, but a proton that may be involved is shown bound to a H_2-evolution site. Centre Y at the −4 oxidation level is required by Mössbauer data (Smith & Lang, 1974), which showed that the e.p.r.-silent Fe atoms in Kp1 protein are all ferrous. There is no precedent for this oxidation level in Fe–S proteins, but it has been detected transiently in polarographic studies on model Fe_4S_4-cluster complexes (Holm & Ibers, 1977). Centre X in species (*a*) is reduced to the −2 oxidation level to produce species (*b*) by a rapid single-electron transfer from Kp2 protein in an MgATP-dependent reaction ($k = 30s^{-1}$ at 10°C; R. N. F. Thorneley, unpublished work). Species (*b*) is e.p.r.-silent and in the steady state accounts for 95% of Kp1 protein. Consequently signals I_a and I_b are 95% bleached (Smith *et al.*, 1973).

Conversion of species (*b*) *into* (*c*) *and species* (*c*) *into* (*a*). Conversion of species (*b*) to species (*c*) must be slow relative to other steps in this H_2-evolving cycle in order that species (*b*) can accumulate to 95% of Kp1 protein in the steady state. A second MgATP-dependent electron transfer from Kp2 protein yields species (*c*). This is a hydride-bound intermediate with centre Y oxidized to the −3 level, giving rise to signal V. The hydride species (*c*) is hydrolysed to



Scheme 1. *H_2 evolution under Ar*

X and Y designate two Fe_4S_4 clusters in Kp1 protein. The Roman numerals below the cluster refer to the e.p.r. signal arising from that cluster under various conditions (Table 1). Signals I_a and I_b arise from centre X and are associated with different pH forms of the protein. The oxidation level (−1, −2, −3 or −4) and the spin state ($S = 0, \frac{1}{2}$ or $\frac{3}{2}$) are indicated for each cluster in intermediate forms of Kp1 protein. Species (*b*) is e.p.r.-silent and accounts for 95% of Kp1 protein under turnover conditions in the absence of CO in both Schemes 1 and 2. Schemes (*a*) and (*b*) are alternative Schemes with signal V arising from the −3 and −1 oxidation levels respectively. $Cys_4 = 4$ cysteines in the peptide chain(s).

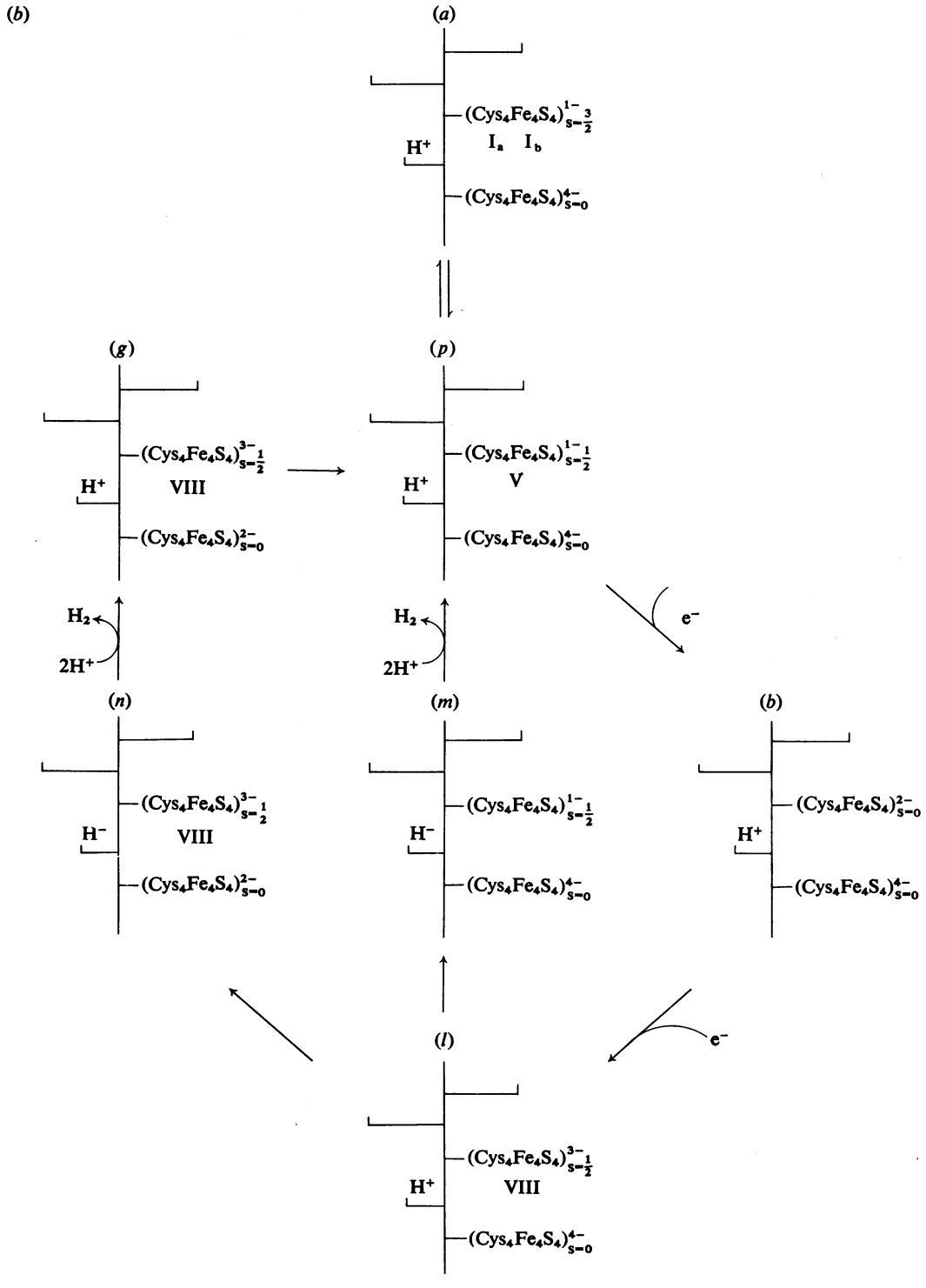
evolve H_2 with coupled internal electron transfer from centre X to centre Y. The accumulation of species (*a*) detected by Mössbauer (Münck *et al.*, 1975) and e.p.r. spectroscopy (Smith *et al.*, 1973) for a system that is allowed to exhaust the supply of dithionite ion is explained by this step.

We have tentatively assigned signal V to centre Y in species (*c*), since this signal is observed under Ar alone after 30min. However, the situation is more complex, since signal V increases with time (R. N. F. Thorneley, R. R. Eady & D. J. Lowe, unpublished work) when the system is evolving H_2 at a linear rate (Thorneley & Eady, 1977). Centre Y at the −3 oxidation level responsible for signal V may be derived from species in Scheme 2 (e.g. isomerization of species *g*), since all the e.p.r. signals of species in this cycle increase in intensity with a time constant approximating to that of the lag for acetylene reduction (R. N. F. Thorneley, R. R. Eady & D. J. Lowe, unpublished work).

Scheme 1(*b*)

Equilibrium of species (*a*) *with* (*p*). Species (*a*) is Kp1 protein as isolated (see description of Scheme 1*a*). Species (*p*) is derived from species (*a*) by a change in spin state of centre X causing signals I_a and I_b to be converted into signal V. Thus signal V in this scheme arises from centre X at the −1 oxidation level.

Conversion of species (*p*) *into* (*b*) *and species* (*b*) *into* (*l*). These two reactions are consecutive one-electron reduction steps in which centre X is reduced


(b)
(a)
$(Cys_4Fe_4S_4)^{1-}_{S=\frac{3}{2}}$
H^+
I_a I_b
$(Cys_4Fe_4S_4)^{4-}_{S=0}$
(g)
(p)
$(Cys_4Fe_4S_4)^{3-}_{S=\frac{1}{2}}$
VIII
$(Cys_4Fe_4S_4)^{2-}_{S=0}$
$(Cys_4Fe_4S_4)^{1-}_{S=\frac{1}{2}}$
V
H_2
$2H^+$
e^-
(n)
(m)
(b)
H^-
$(Cys_4Fe_4S_4)^{2-}_{S=0}$
(l)

from the −1 to the −3 oxidation level, resulting in the appearance of signal VIII. In the steady state, as in Scheme 1(*a*), more than 90% of the Kp1 protein is present as the e.p.r.-silent species (*b*).

Hydride formation. Species (*m*) or (*n*), which both have a bound hydride ion, are formed from species (*l*) by the transfer of two electrons from centre X or centre Y respectively to the bound proton. In species (*m*) signal V arises from centre X at the −1 oxidation level.

H_2 evolution. Hydrolysis of the bound hydride ion in species (*m*) or (*n*) results in H_2 evolution and the formation of species (*p*) or (*g*). Species (*p*) is also formed from species (*g*) by redistribution of two electrons from centre X to centre Y. Note that species (*g*) appears in both Schemes 1(*b*) and 2 and may be involved in the coupling of these two cycles.

Scheme 2: acetylene reduction, ethylene binding and CO inhibition

Equilibrium (*d*) ⇌ (*h*) (*binding of ethylene*). Ethylene binds preferentially to Kp1 protein with centre X in the spin $S = \frac{1}{2}$ state and centre Y at the e.p.r.-silent −2 oxidation level. This accounts for the decrease in signal VI and the corresponding increase in signal IV intensity as acetylene displaces ethylene via the coupled equilibria between species (*h*), (*d*), (*e*) and (*f*).

Equilibrium (*d*) ⇌ (*e*). This is an internal redox reaction in which an electron is transferred from centre Y to centre X. The relative amounts of species (*d*) and (*e*) depend on the redox potentials of these two centres. This accounts for the appearance of signal IV at the expense of signal VI and allows signals IV and VI to be associated with different Fe_4S_4 clusters, as suggested by their e.p.r. parameters.

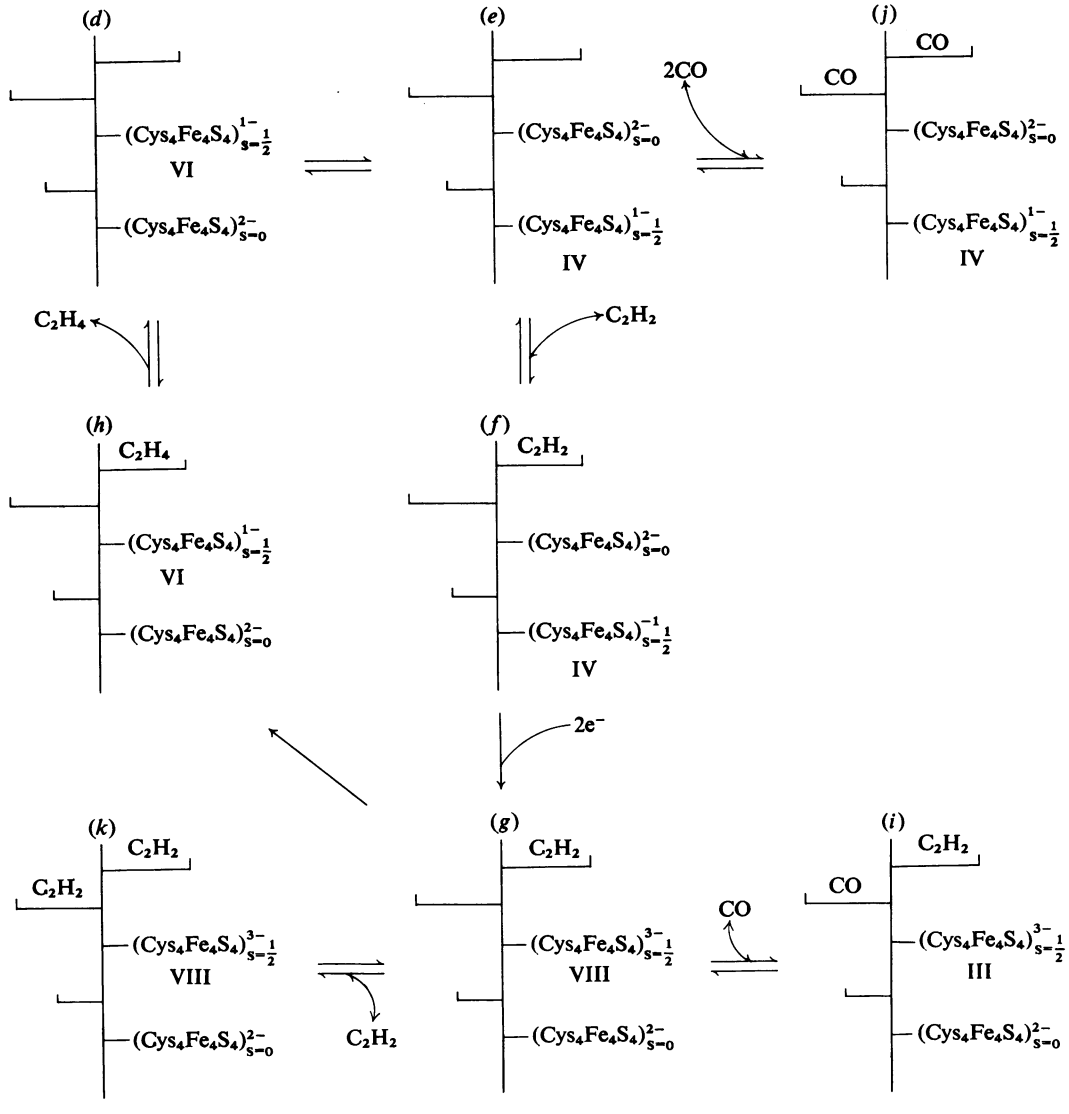
Equilibrium (*e*) ⇌ (*f*) (*tight acetylene binding*). Acetylene binds preferentially to Kp1 protein with centre X reduced to the e.p.r.-silent −2 level and centre Y oxidized to the −1 level. This explains the increase in signal IV on acetylene binding and the competitive nature of acetylene binding relative to ethylene, owing to the coupled equilibria between species (*h*), (*d*), (*e*) and (*f*).

Reduction of species (*f*) *to* (*g*). Centres X and Y are reduced to the −3 and −2 oxidation levels respectively by the transfer of two electrons either from other centres in Kp1 protein or from Kp2 protein in an MgATP-dependent reaction. This allows signal VIII to arise from a reduced centre X, and centre Y to be e.p.r.-silent. Form (*g*) reduces acetylene to ethylene (step *g* to *h*), binds CO tightly (low-CO form) in step (*g*) to (*i*) and binds acetylene weakly (step *g* to *k*).

Oxidation of (*g*) *to give* (*h*) (*acetylene reduction*). Acetylene is reduced by a two-electron transfer process, which is coupled to *cis*-protonation to give ethylene (Kelly, 1969). Centre X is oxidized to the −1 oxidation level by the transfer of two spin-paired electrons. The transfer of two electrons with parallel spins would result in the formation of a diradical in an energetically unfavourable reaction. One-electron reduction would result in free-radical intermediates, which are not consistent with the observed *cis*-deuteroethylene formation for acetylene reduction in ^2H_2O (Kelly, 1969). The species giving rise to signal I_c could be formed by a spin-state change of centre X from $S = \frac{1}{2}$ to $S = \frac{3}{2}$ in species (*d*) or (*h*). Thus whether centre X at the −1 oxidation level with $S = \frac{3}{2}$ gives signals I_a and I_b or signal I_c is determined by whether centre Y in that species is at the −4 or −2 oxidation level.

Equilibrium (*g*) ⇌ (*k*) (*weak acetylene binding*). At high concentrations acetylene binds to the high-affinity CO-binding site, causing signal VIII to increase as signal IV decreases (see Fig. 5). The binding of acetylene to the site does not induce the small conformation change produced by CO binding, which distinguishes forms (*g*) and (*k*) from (*i*), i.e. signal VIII, not signal III, increases. Acetylene is probably not reduced at this site, since CO is a non-competitive inhibitor of acetylene reduction in Kp nitrogenase (B. E. Smith & A. Funnell, unpublished work), and no evidence was obtained for a corresponding species with bound ethylene. However, Davis *et al.* (1978) deduced high- and low-affinity acetylene-reducing sites with apparent K_m values of 0.003 atm and 0.23 atm at 30°C for Cp nitrogenase from steady-state kinetic data for ethylene formation. The low-K_m form was easier to detect when Cp2 protein was limiting and the high-K_m form was easier to detect when Cp1 protein was limiting. This observation supports Scheme 2, since limiting amounts of Fe–protein will favour the oxidized tightly bound acetylene species (*f*) and limiting Mo–Fe protein the more reduced weakly bound acetylene species (*k*). This also suggests that the reduction of species (*f*) to (*g*) is achieved by electron transfer from Fe protein and not from other centres in the Mo–Fe protein. Shah *et al.* (1975) reported two acetylene-binding sites; however, in their case binding to the low-affinity site caused inhibition of substrate reduction. All these data agree with our findings that high- and low-affinity acetylene-binding sites exist, but also indicate a species variation with respect to the role of the second site.

Equilibrium (*g*) ⇌ (*i*) (*tight CO binding*). Form (*g*) of Kp1 protein is converted into form (*i*) by tight stoicheiometric binding of CO. The binding of CO is non-competitive with respect to acetylene. Form (*i*) cannot reduce acetylene. A small conformation change which perturbs centre X when CO binds accounts for the slight differences in the e.p.r. parameters for signals VIII and III. This is consistent with the assignment of the low-CO signal



Scheme 2. *Acetylene reduction and CO inhibition*

For a description of the terms used see Scheme 1. The catalytic cycle for acetylene reduction involves species (*d*), (*e*), (*f*), (*g*) and (*h*). Species (*i*) and (*j*) are dead-end complexes of CO with Kp1 protein. Species (*k*) is written as a dead-end bis-acetylene complex. This species may be involved in acetylene-reduction cycles involving one or both of the bound acetylene molecules. These cycles together with a number of equilibria involving unliganded species [i.e. (*g*) and (*i*) with no bound acetylene] have been omitted for the sake of clarity. Only e.p.r.-active intermediates have been included.

to a reduced Fe_4S_4 cluster (Davis *et al.*, 1978). Forms (*g*) and (*i*) may also exist in the absence of acetylene, since we have no evidence to suggest that they are substrate-induced.

Equilibrium (*e*) $\rightleftharpoons$ (*j*) (*weak CO binding*). This accounts for the increase of signal IV at high partial pressures of CO. Form (*j*) has centre Y at the −1 oxidation level and centre X at the e.p.r.-silent −2 level. Davis *et al.* (1978) have suggested that this type of signal is due to an oxidized Fe_4S_4 cluster. For the concentration of species (*j*) to increase at high partial pressures of CO, it must bind to a second

site. We have used the acetylene-binding site to minimize the total number of binding sites used in the Scheme. A metal centre that will bind acetylene will probably also bind CO. Since acetylene reduction is inhibited at low partial pressures of CO by the accumulation of form (*i*), the second site of CO binding would not be detected in inhibition studies involving acetylene reduction.

Coupling of the H_2-evolution cycle to the acetylene-reduction cycle

The e.p.r. signals associated with species (*d*), (*e*), (*f*), (*g*), (*h*) and (*k*) appear during the lag phase and reach a maximum intensity when the rate of acetylene reduction has become constant (R. N. F. Thorneley, D. J. Lowe & R. R. Eady, unpublished work). The two species with bound CO, (*i*) and (*j*), are only observed under turnover conditions and appear over a period of about 10s at 30°C with Cp nitrogenase (Davis *et al.*, 1977). We suggest that the slower coupling between the two cycles in Schemes 1 and 2 limits the rate of appearance of signals arising from species with both CO and acetylene/ethylene bound, i.e. a common process accounts for the lags observed in both acetylene reduction and CO binding. Since this slow coupling may either be a consequence of a slow covalent modification (e.g. phosphorylation, adenylylation, as suggested by Smith *et al.*, 1976*a*) or of the kinetic complexity of the system, we do not feel justified in writing a mechanism at the present time. However, since in the presence of CO up to 50% of Cp1 protein accumulates in species (*i*) or (*j*), whereas H_2 evolution is uninhibited, the cycle in Scheme 2 must also be capable of proton reduction by species (*i*) to give (*j*). This also explains inhibition of H_2 evolution by acetylene occurring only after the lag phase (Thorneley & Eady, 1977).

These schemes for H_2 evolution, acetylene reduction and CO inhibition utilize intermediates derived from two of the three types of Fe–S clusters detected for Kp1 protein by Mössbauer spectroscopy (Smith & Lang, 1974). The ability of Mössbauer spectroscopy to assign all the Fe atoms in Kp1 protein to various clusters and oxidation states is restricted to intermediates present at high concentrations in the steady state. In the present paper we have demonstrated the ability of the e.p.r. technique to detect intermediates present in low concentration and together with the earlier Mössbauer studies described above to propose a working hypothesis for the mechanism of nitrogenase.

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