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EVIDENCE FROM MOSSBAUER SPECTROSCOPY FOR THE ROLE OF IRON IN NITROGEN FIXATION

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SUMMARY

The two nitrogenase proteins of *Klebsiella pneumoniae* were prepared from bacteria grown in ^{57}Fe-enriched medium. The Mossbauer spectrum of the FeMo protein in weak dithionite exhibited a broad paramagnetic component in addition to a narrow quadrupole doublet. The paramagnetic intensity could be reduced by removal of dithionite and by incubation in strong dithionite. This is interpreted in terms of reducible Fe atom pairs whose members are close enough to permit spin coupling and the sharing of electron spin. The Fe protein in weak dithionite exhibited a narrow doublet, probably ferric, in addition to a weak high spin ferrous signal with isomer shift 1.4 mm/sec, splitting 2.8 mm/sec. Mixtures of the two proteins yielded simple sum spectra unless both ATP and $Na_2S_2O_4$, the ingredients required for active reduction, were present. Under this condition an unusual signal appeared, with isomer shift 0.65 mm/sec, quadrupole splitting 3.05 mm/sec and area about 10 % of that of the FeMo spectrum. At the same time the area of the high spin ferrous signal varied with substrate and was especially intense in the presence of CO. Variation of spectra with substrate and inhibitor appeared to involve changes in the relative intensities of spectral components, but not a change in their individual shapes.

$^{14}CN^-$ was bound specifically only in the presence of both proteins, $Na_2S_2O_4$, and ATP; CO prevented this binding.

NO rapidly and irreversibly inactivated the Fe protein; the FeMo protein was more slowly inactivated and Mossbauer spectra in the presence of NO did not show the paramagnetic spectrum characteristic of Fe–nitrosyl derivatives. The multiple roles of Fe in nitrogen fixation are considered and a hypothesis for the method of nitrogen fixation based on the Mossbauer data is given.

INTRODUCTION

Evidence that Fe (ref. 1) and Mo (ref. 2) are required by microorganisms fixing nitrogen was obtained before reproducible cell-free fixation was reported with *Clostridium pasteurianum*[3]. Subsequently, nitrogenase from a number of bacteria has been partially purified and separated into two components; for each nitrogenase, one

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protein contained Fe and Mo, the other contained Fe. Zn has also been reported in the FeMo protein[4]. Research by a number of workers has established that in addition to the two proteins, nitrogen fixation requires an ATP and electron supply and that other small molecules may be reduced including acetylene, azide, cyanide or isocyanide; all these substrates can form metal complexes[5] but there is little direct evidence for the role of Fe in the nitrogenase.

Different research groups have reported varying amounts of Fe in each protein but the FeMo protein probably contains not fewer than 8 atoms Fe/molecule of protein and the Fe protein not fewer than 2 atoms Fe/molecule of protein. Some of the Fe atoms may function as electron carriers like ferredoxin, indeed the similarities of the two proteins with ferredoxin (non-haem Fe, labile sulphur, oxygen sensitivity) has led MORTENSON *et al.*[6] to name them molybdo- and azo-ferredoxin, respectively. Some of the Fe atoms may play a part in binding nitrogen (*cf.* oxygen binding to haemoglobin[7]) and a two-metal site for reduction of nitrogen was suggested by WINFIELD[8] in 1954. Other mechanisms for nitrogen reduction involving Fe have been suggested[9] including one, based on a chemical model[10], where an Fe–N–Mo bridged complex is postulated to form during biological reduction of nitrogen to ammonia.

Mossbauer spectroscopy[11], which has already provided important information about Fe in various biological systems such as ferredoxin[12], haemoglobin[13] and other haem proteins[14], should prove a very useful additional procedure for investigating nitrogenase, in particular since it is very specific, able to distinguish oxidation states of Fe and may give useful information about the ligands binding Fe and changes in these which may occur during reaction of the two proteins, ATP and $Na_2S_2O_4$.

Some Mossbauer work on nitrogenase has already been published by Russian workers. In one case whole cells of *Azotobacter vinelandii* were used[15] containing other Fe-containing proteins, *e.g.* a non-haem Fe protein[16] and cytochromes which would contribute to the spectra and make interpretation difficult. The FeMo protein from the same organism was investigated[17] but under conditions of high $Na_2S_2O_4$ or $NaHCO_3$ concentration.

In this work we present results with ^{57}Fe-enriched nitrogenase prepared from *K. pneumoniae* which fixes nitrogen anaerobically. Under these conditions cytochromes are not produced. The nitrogenase was separated into its two proteins and examined in a variety of combinations including FeMo protein or Fe protein alone or together, with or without ATP or substrate.

MATERIALS AND METHODS

Preparation of $^{57}FeSO_4$

100 mg of ^{57}Fe-enriched Fe was obtained from the United Kingdom Atomic Energy Commission, Harwell. The sample, which had mass analysis 0.25 % ^{54}Fe, 12.8 % ^{56}Fe, 86.5 % ^{57}Fe and 0.43 % ^{58}Fe, was dissolved in excess dilute H_2SO_4 by gently warming. No pH adjustment of the $^{57}FeSO_4$ was made since the culture medium to which the Fe was added was sufficiently buffered to neutralise the excess acid.

Culture of K. pneumoniae

This organism was grown on the nitrogen-free medium described by HINO AND WILSON[18] for preparation of ^{56}Fe nitrogenase (containing ^{57}Fe at the natural abundance level). Cells were collected and stored as described previously[19].

Since Fe has a number of stable isotopes of natural abundance: ^{54}Fe, 5.9 %; ^{56}Fe, 91.5 %; ^{57}Fe, 2.24 %; ^{58}Fe, 0.33 %; and only ^{57}Fe gives Mossbauer spectra, nitrogenase from *K. pneumoniae* grown with commercial Fe salts would give very poor spectra. Therefore cells were grown on a medium containing $^{57}FeSO_4$ after treatment of the components to reduce the level of contaminating ^{56}Fe in chemicals and on glassware. Solution A containing 400 g sucrose, 10 g $MgCl_2 \cdot 6\,H_2O$, 2 g NaCl in 1 l and Solution B containing 240 g K_2HPO_4, 68 g KH_2PO_4, 200 μg *p*-aminobenzoic acid and 100 μg biotin also in 1 l were treated separately with 8-hydroxyquinoline in chloroform to extract residual Fe in the chemicals using the procedure of WARING AND WERKMAN[20]. All glassware used was treated with concentrated H_2SO_4 followed by treatment with 8-hydroxyquinoline in chloroform. The preliminary acid treatment was particularly necessary for the gas sparger of the growth vessel since its sintered glass disc tended to become heavily contaminated with Fe which was not dislodged by routine washing procedures.

After treatment, Solution A was diluted in 20-l culture vessel with 18 l of glass-distilled water and autoclaved. Solution B was autoclaved separately and added aseptically to Solution A on cooling. 100 ml of this Fe–Mo-deficient medium were transferred aseptically to a sterile 250-ml medicine bottle containing a glass bubbler. Sterile $NaMoO_4$ and $^{57}FeSO_4$ were then added to the 20 l of medium to give a final concentration of 2 mg/l of Mo and 1 mg/l of Fe. (The usual concentration of Fe in the medium of HINO AND WILSON[18] is 3 mg/l.)

Six 100-ml amounts of the complete medium were transferred aseptically into 250-ml sterile medicine bottles equipped with bubbling tubes. One of these bottles was innoculated with 0.1 ml of *K. pneumoniae* culture grown on nutrient broth. Good growth was obtained after 3 days incubation at 30° with sterile nitrogen bubbling through the culture. 0.1 ml of this culture was transferred to a fresh 100 ml of complete medium and the growth and subculture procedure repeated to reduce the level of ^{56}Fe in the bacteria to a minimum. When 0.1 ml of the third subculture was added to the 100 ml of Fe–Mo-deficient medium only very slow growth of bacteria occurred. Even when sufficient Mo was added, the growth rate remained very slow, indicating that the incomplete 8-hydroxyquinoline-treated medium was deficient in Fe. Most of the third subculture was used to inoculate the 20-l pot which was then sparged at 30° with nitrogen. The cells were harvested from the culture after 48 h, before growth had stopped but when the rate of acetylene reduction per mg dry weight of cells was greatest. A total of 80 l of ^{57}Fe-enriched cells were grown in this way producing a combined yield of about 250 g wet paste of cells.

Preparation of nitrogenase proteins

The procedures used for the preparation of crude nitrogenase, its separation into FeMo protein and Fe protein by chromatography on DEAE-cellulose and the further purification of each by repeated chromatography on DEAE-cellulose at a lower pH have been described[19]. Each protein was concentrated after purification by pressure filtration through a semipermeable membrane so that it was sufficiently concentrated with respect to Fe to give satisfactory Mossbauer spectra. The concentrated protein solutions were centrifuged separately under argon to sediment small amounts of denatured protein and each clear supernatant was drawn into an argon-flushed syringe, then frozen and stored in bead form in liquid nitrogen.

The absorption spectrum of each was similar to those published for the corresponding proteins from *A. vinelandii*[21] or *C. pasteurianum*[22]. Useful electrophoretic studies of Fe protein were not possible since it was very rapidly irreversibly inactivated. The behaviour of the FeMo protein on electrophoresis has been described[23].

The FeMo and Fe protein of nitrogenase were analysed for Fe, Mo and Zn by atomic absorption spectrophotometry. The FeMo protein contained 2.5 μg Fe/mg protein and 0.44 μg Mo. Assuming 1 Mo/molecule of protein the FeMo protein had a molecular weight of about 216000 and a ratio of 10 (9.7) Fe atoms to 1 Mo atom. The Fe protein contained no detectable Mo and 1.9 μg Fe/mg of protein. Using a molecular weight of about 60000 calculated from the protein's R_F on Sephadex G-75, this protein has 2 atoms Fe/molecule of protein. Both proteins also contained Zn but, since other proteins examined also contained Zn, this metal was probably bound non-specifically.

Preparation of Mossbauer samples

All Mossbauer spectra were run on samples contained in holders having a working volume of about 0.7 ml. For samples of the FeMo or Fe protein alone, each was thawed out under argon, warmed to about 20° and syringed into a sample holder gently flushed with argon or other gas. When required, about 4 mg of solid $Na_2S_2O_4$ were added at this stage and 0.1 ml of an ATP mixture containing 10 μmoles ATP; 40 μmoles creatine phosphate; 50 μmoles Tris–HCl (pH 7.4); 10 μmoles $MgCl_2$ and 1 mg creatine kinase. 0.05 ml of 10 mM freshly prepared solution of the substrate KCN was also added to the holder when required.

The protein sample was withdrawn into a 2-ml syringe and re-injected into the gas-flushed sample holder. The withdrawal re-injection cycle was repeated several times to ensure complete mixing of all the liquid components, solution of gas and time for any reaction to reach a steady state. This procedure was continued for about 90 sec. The sample in the holder was then rapidly frozen by dipping into liquid nitrogen whilst a stream of the appropriate gas was blowing onto the surface; once solid the sample was stored immersed in liquid nitrogen.

For samples containing both FeMo and Fe protein, equal volumes of each were combined in a 6-ml bottle under argon before starting the injection–withdrawal–re-injection cycle into the holder under the appropriate gas and then freezing as described above. Unless stated otherwise, the amounts of protein used per holder were: FeMo protein alone 56 mg, Fe protein alone 28 mg and in combination 28 and 14 mg, respectively.

Assay of nitrogenase

The procedures used for assay of nitrogenase activity by measuring the rate of acetylene reduction have been published[19]. Where only FeMo protein was present in a Mossbauer sample it was assayed by adding to excess of Fe protein; similarly Fe protein was assayed by adding excess FeMo protein. Reduction in samples containing both proteins, $Na_2S_2O_4$ and ATP *plus* the substrate CN^- or acetylene was demonstrated directly since after freezing the sample, methane (from CN^-) or ethylene (from acetylene) was detected by gas chromatographic analysis of the residual gas in the syringe. However, for quantitative measurements, acetylene reduction was determined with small samples removed from Mossbauer holders containing the systems;

FeMo protein alone, Fe protein alone and the various combinations, before these were frozen. Measurements on twelve different samples before freezing and on the same system thawed out after Mossbauer spectroscopy showed no significant inactivation of nitrogenase was caused by any of the procedures used except by NO gas (see RESULTS).

Further details of procedures and sources of gases and chemicals are given in refs. 24 and 25.

Counting of Mossbauer samples

Mossbauer measurements were made on a constant acceleration Mossbauer spectrometer of the usual type in connection with a multichannel analyzer operating in the time mode[26]. The γ-ray source was ^{57}Co diffused into Pd foil, and had a linewidth of 0.20 mm/sec when used with a thin Fe absorber. Calibrations were made with room temperature Fe foil[27], and velocities are relative to the centre of its spectrum. Samples were contained in polythene holders of volume 0.7 ml, with sample thickness 1/8 inch in the beam direction. Counting rates were of order $2 \cdot 10^4$/sec of which typically 85 % were 14.4 keV γ-rays and most of the remainder resulted from compton scattering of the more energetic γ-rays in the argon–methane counter gas. The background rate was determined at the start of each run by inserting an aluminium filter in the γ-beam, and the absorption scales on the figures are background corrected. In the 4.2°K runs the samples were immersed in liquid He, while in the 77°K runs they were in N_2 gas at atmospheric pressure. Runs at 195°K were in air at atmospheric pressure. Small fields were applied with permanent magnetron magnets, and fields up to 5.5 kgauss were obtained with an electromagnet. Small fields (approx. 100 gauss) aligned along the γ-beam were obtained at 4.2°K by inducing persistent currents in Pb washers concentric with the absorber in a Helmholtz arrangement. Transfer of samples from the liquid nitrogen storage Dewar to the spectrometer Dewars involved only a few seconds exposure to ambient temperature.

$^{14}CN^-$ binding to nitrogenase

In these experiments nitrogenase was dialysed under argon or CO against $K^{14}CN$ with or without an ATP supply and samples were removed at intervals to determine the $^{14}CN^-$ associated with dialysing buffer or protein. Though nitrogenase, $Na_2S_2O_4$ and ATP reduced CN^- to methane, the rate of reduction dropped sharply with temperature; at 10° it was 15 % of the rate observed at 30 and at 0° the reduction was not detected after a 1-h incubation period.

Fig. 1 shows the arrangement of apparatus used for equilibrium dialysis.

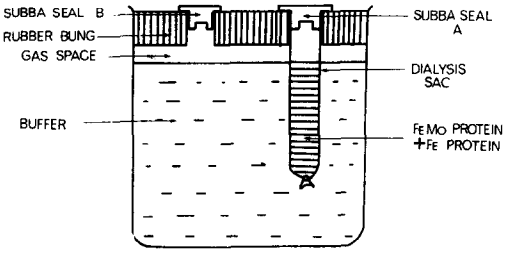

Fig. 1. Apparatus used for equilibrium dialysis with $^{14}CN^-$.

8/32 inch dialysis tubing, knotted at one end and sealed at the other with a subba seal (A) set into a large rubber bung, was suspended in 20 ml of 25 mM Tris–HCl buffer (pH 7.4) containing 5 mg creatine kinase, 400 μmoles creatine phosphate and 200 μmoles $MgCl_2$. After flushing with either CO or argon the system was tightly sealed with the bung and 400 μmoles of $Na_2S_2O_4$ were injected into the buffer through subba seal B in the rubber bung. The mixture was allowed to equilibrate for 1 h at 0° before injecting 1 ml of solution containing 10 mg of nitrogenase (FeMo protein *plus* Fe protein) at 0° into the dialysis sac through subba seal A. $K^{14}CN$ was then injected through subba seal B to give a final concentration of 50 μM in the buffer and the system was mixed by gently rotating. Samples were withdrawn at intervals from both inside and outside the dialysis tubing using argon flushed syringes with long needles that could be inserted through subba seal A or B. Details of counting radioactivity by end window counter have been described[23]. After 7 h 200 μmoles ATP were injected if required into the buffer and further samples were removed at intervals from inside and outside the dialysis tubing for counting. At the end of the dialysis period (22 h) the activity of the nitrogenase was re-checked by assaying the rate of acetylene reduction. No significant loss of activity had occurred during the experiment and the dialysing buffer still reduced benzyl viologen after 22 h, confirming the presence of $Na_2S_2O_4$. The production of methane after incubation of 5 ml of the buffer with nitrogenase, ATP and $Na_2S_2O_4$ at 30° confirmed the presence of CN^- at the end of dialysis.

General discussion of Mossbauer spectroscopy

The Mossbauer measurements are always made in the frozen state, while we wish to relate them to the function of the nitrogenase in liquid solution near room temperature. We may hope that the freezing enables us merely to preserve for leisurely study the exact instantaneous situation in the room temperature solution, but we should not be surprised to find that nature is not so kind. The behaviour of haem proteins, particularly the high spin–low spin mixtures[14], is known to be freezing-rate dependent, and similar effects could occur in the present system. We have also the complication that many of the substrates of interest are gaseous, and their solubility will decrease strongly on freezing. Thus while the Mossbauer measurements provide a wealth of new insights into the state of Fe in nitrogenase, the conditions under which they are made should always be borne in mind.

In the paragraphs which follow we will attempt a rough interpretation of the Mossbauer spectra in terms of the electronic environment of the Fe nuclei, *i.e.* in terms of the chemical bonding situation in the immediate neighbourhood of the Fe. As a result of the efforts of many investigators, a large body of relevant information is now available. The simplest Mossbauer spectra occur when magnetic hyperfine interaction is absent, and electrostatic interactions give rise to two absorption lines. These are usually characterized by their energy separation, ΔE, and by δ, the energy of their centre of symmetry. The γ-ray energy is varied by moving the source and energy is commonly measured in units of source velocity. ΔE, the quadrupole splitting, is a measure of the electronic charge asymmetry near the Mossbauer nucleus, while δ, the isomer shift, is a measure of the electronic charge density within the nuclear volume. High spin ferrous iron, Fe^{2+}, is the most easily identifiable species, having δ usually in the range 1.0–1.5 mm/sec, and ΔE in the range 2.0–3.7 mm/sec. ΔE is

commonly temperature dependent in the range below room temperature. Low spin ferrous, Fe^{II}, characteristically has δ between -0.4 and 0.4 mm/sec, and ΔE less than 1 mm/sec. High spin ferric, Fe^{3+}, tends to lie in the isomer shift range 0.3–0.8 mm/sec, with ΔE less than 1.5. For low spin ferric, Fe^{III} δ tends to lie in the range -0.2 to 0.2 mm/sec, with ΔE temperature dependent and seldom greater than 2 mm/sec. There are exceptions to these rules, particularly biomolecules (for example Fe^{II} oxyhaemoglobin with $\Delta E = 2.2$ mm/sec, ref. 13) and confusion among the last three types can easily occur. Magnetic interactions can provide additional information[28]. Fe^{II} has no unpaired spin and no significant internal effective magnetic field is seen. Fe^{2+} has unpaired spin, but usually has a singlet ground state. This exhibits an internal effective magnetic field only in the presence of an applied field. Ferric iron, with half integral electron spin, has all electronic states magnetic. In concentrated materials rapid spin–spin interaction tends effectively to eliminate the magnetic hyperfine interaction, but in magnetically dilute ferric materials it is seen at low temperatures. For example, all ferric haem proteins which have been examined have low-temperature spectra with pronounced magnetic features. We would expect the Fe in nitrogenase solutions to be magnetically dilute as between molecules, but we have no *a priori* knowledge of the arrangement of the Fe atoms within a single molecule. In spite of the considerable information available from Mossbauer spectra, exotic situations do arise and sometimes the interpretation is ambiguous. One should not be surprised if the Fe involved in biological nitrogen fixation is unusual, for this is a rather unusual process. Our problem is further complicated by the presence of a number of inequivalent Fe sites in nitrogenase, with overlapping Mossbauer spectra. We emphasize therefore that the interpretations given below are somewhat tentative, while we hope and expect that most are correct.

RESULTS

Figs. 2–9 show the essential results of over a hundred Mossbauer measurements on thirty-five nitrogenase specimens. In most cases results were checked by examining a second sample. The only inconsistencies which were observed were those mentioned below associated with the difficulty of freezing fixing mixtures before ATP or substrate were exhausted.

Mossbauer spectra of the separated proteins

The nitrogenase proteins are normally prepared and stored in weak dithionite solution. We have found that the spectra of the FeMo protein in weak dithionite solution are not affected by the addition of N_2 or ATP, but vary with dithionite concentration. In Fig. 2 are shown the helium-temperature spectra under a variety of conditions. At (a), (b), and (c) are the spectra of the protein in dilute dithionite. The wide component is obviously magnetic in origin, and its occurrence in zero applied field is indicative of half integral electronic spin, hence of ferric iron. Application of a magnetic field, at (b), sharpens the lines without significantly moving them, and a comparison of (b) and (c) indicates that at least the strong magnetic lines are not sensitive to field direction. This suggests that the magnetic hyperfine interaction is diagonal, *i.e.* that the operators S_+ and S_- have zero matrix elements within the electronic doublet. The effective electron spin must therefore exceed $\frac{1}{2}$, although

the width of the spectrum is considerably smaller than would be expected from a high spin ferric complex. Fields of 5.5 kgauss had negligible effect on the spectrum, implying that the doublets of the spin multiplet are separated by a zero field splitting of at least several cm^{-1}. The spectrum of Fig. 2(d) corresponds to a sample in which

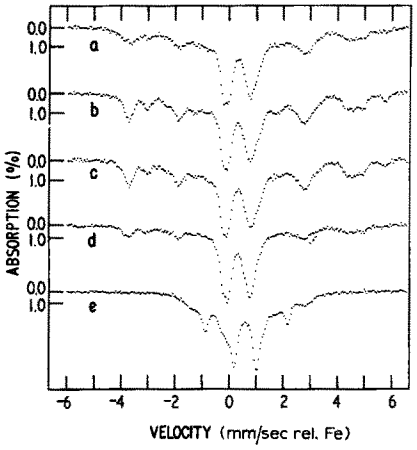

Fig. 2. Spectra at 4.2 °K of the FeMo protein of nitrogenase. The first three are from an argon-flushed sample containing 4 mg of $Na_2S_2O_4$ with (a) no magnetic field, (b) a field of about 100 gauss parallel to the γ-beam, and (c) a field of 550 gauss transverse to the γ-beam. Removal of dithionite by dialysis results in spectrum (d), obtained in 550 gauss transverse field. The sample responsible for (e) in 550 gauss was undialysed material incubated for 1 h under argon at 20°, with 20 mg of dithionite per ml of solution.

dithionite has been removed by dialysis under argon. It is clear that the relative strength of the wide magnetic part of the signal has been reduced. This behaviour must be considered unusual, for removal of reductant normally tends to take an Fe complex toward the half integral spin (ferric) state. One possible explanation is that we are dealing with a transition between ferric iron and Fe^{IV}. An objection to this is that the isomer shift of Fe^{IV} would be roughly 0.0 mm/sec (ref. 29) and we find no such spectral component. A second explanation, and the one which we favour, is that the Fe atoms occur in multiples, probably pairs, which can spin couple. Thus in the complete absence of reductant we might expect to see ferric complexes with their spins coupled anti-parallel in pairs to give a combined state which has no net spin. Addition of reductant could then add an electron to the pair, giving rise to a Kramers doublet which must be magnetic by definition. We know that the magnetic state has unpaired spin greater than just that of the added electron. The small value of effective magnetic field at the Fe nuclei and the large intensity of the magnetic signal could be explained if the unpaired spin were to be shared between the two Fe sites. A somewhat similar behaviour is known for the FeS proteins, where magnetic properties as exhibited by ESR[30] and Mossbauer spectroscopy[12] occur in the reduced state, but disappear in the oxidized state of the system. The situation is not exactly parallel, however, because the effective spin is ½ in that case, while it is clearly greater in the present one. The intensity of the magnetic part of the spectrum would be maximum when each Fe pair had acquired an unpaired electron. We can extend the picture and postulate that addition of another electron could return the pair to

an integral spin state. The spectrum of Fig. 2(e) corresponds to FeMo protein which has been incubated for 1 h at 20° under argon in a solution containing 20 mg of dithionite per ml. The wide magnetic spectrum has disappeared, and a number of new features have appeared in the spectrum. This may possibly correspond to the process postulated above. No such second stage of reduction has been shown in the FeS proteins, but the difficulty sometimes experienced in performing reductions and the occasional production of a new species, thought to be denatured, could be indications that it is occurring.

It appeared possible that fully reduced FeMo protein might make either ATP or Fe protein unnecessary for substrate reduction. However no ethylene was produced when a mixture of 20 mg/ml of $Na_2S_2O_4$, acetylene, and FeMo protein was incubated for 1 h alone, with ATP, or with Fe protein.

We give our magnetic spectral component the name M1 (for FeMo protein) for future reference and consider the narrow doublet of the first four spectra of Fig. 2. For convenience, we list in Table I all spectral components, together with their charac-

TABLE I

TABLE OF SPECTRAL COMPONENTS

Name	*Source*	*Remarks*	*Tentative assignment*
M1	FeMo protein	Wide paramagnetic spectrum at 4.2 °K, with $S > \frac{1}{2}$. Intensity decreases if $Na_2S_2O_4$ is removed. Disappears in fixing mixture and in very concentrated $Na_2S_2O_4$ $\delta = 0.37$ mm/sec, $E = 0.75$ mm/sec, at 77 °K	Ferrous–ferric coupled pairs
M2	FeMo protein	$\delta = 0.35$ mm/sec, $\Delta E = 0.9$ mm/sec at 4.2 °K, little temperature dependence. Area varies with concentration of reductant	Coupled Fe^{3+} pairs, zero net spin
M3	FeMo protein	Precursor of M4	
M4	FeMo protein	$\delta = 0.65$ mm/sec, $\Delta E = 3.05$ mm/sec at 4.2 °K. Little temperature dependence. Present in fixing mixtures. Constant area corresponding to one Fe atom, independence of substrate	Fe^{II} with unusually strong π bonding
M5	FeMo protein	$\delta = 0.6$ mm/sec, $\Delta E = 0.8$ mm/sec at 4.2 °K. Little temperature dependence. Present in fixing mixture. Area represents approx. 4 atoms	Coupled Fe^{3+} pairs with zero net spin. Possibly Fe^{II}
M6	FeMo protein	$\delta = 0.35$ mm/sec, $\Delta E = 0.7$ mm/sec, at 77 °K. Indistinct at 4.2 °K. Area represents approx. 4 atoms	Fe^{III}–Fe^{II} pairs
F1	Fe protein	$\delta = 0.45$ mm/sec, $\Delta E = 1.1$ mm/sec at 77 °K. Little temperature dependence	Fe^{3+} coupled pairs with zero net spin. Possibly Fe^{II}
F2	Fe protein	$\delta = 1.4$ mm/sec, $\Delta E = 2.8$ mm/sec at 77 °K, reducing to 2.4 mm/sec at 195 °K. Enhanced by ATP	Fe^{2+} in asymmetric crystal field. Little doubt
F3	Fe protein	Line at 0.6–0.7 mm/sec, probably also line near 0.0 mm/sec. Area correlates with F2	Fe^{II}

teristics and tentative interpretation. In order to remove the spectrum of M1 we have stripped 2(c) from 2(d) so that the resultant spectrum has minimum mean square signal (*i.e.* absorption) in the regions $v < 2.0$ mm/sec and $v > 4.0$ mm/sec. This, hopefully, should remove M1 and leave the remainder. The resultant is, for the most part, a pair of lines at -0.1 mm/sec and $+0.8$ mm/sec, which we call M2. Its isomer shift of 0.35 mm/sec and its lack of magnetic features would normally suggest that M2 is low spin ferrous. In view of our previous discussion, however, we provisionally assign it to high spin ferric complexes in spin coupled pairs. This is consistent with both isomer shift and quadrupole splitting.

The spectra of the FeMo protein at 77 °K are shown in Fig. 3. The wide magnetic components are missing because at this relatively high temperature interaction with vibrational modes causes the electron spins to relax rapidly, thus drastically reducing the effective magnetic hyperfine interaction. Comparing spectra 3(a) and 3(b) in order to see the forms of M1 and M2 at 77 °K, we find that M2 is essentially unchanged from its 4.2 °K appearance. This is consistent with our Fe^{3+} assignment, for such complexes usually have no low lying orbital excitations and hence have temperature-independent quadrupole splitting. The M1 spectrum at this temperature has lines at 0.0 mm/sec and 0.75 mm/sec, the isomer shift of 0.37 mm/sec being consistent with both Fe^{3+} and Fe^{II}, and not inconsistent with our model, which could in a sense be described as a linear combination of them.

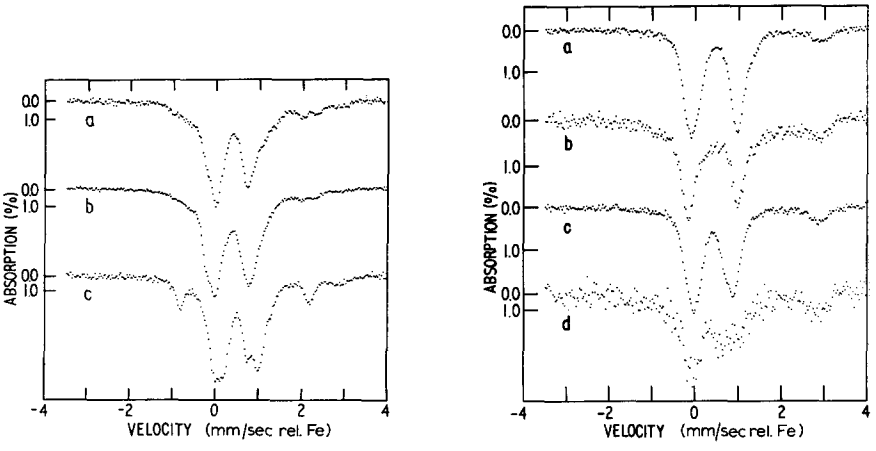

Fig. 3. Spectra of the samples of Fig. 2 but taken at 77 °K in zero field. The conditions are (a) argon flushed, weak dithionite, (b) dithionite removed by dialysis, and (c) incubated under argon in strong dithionite solution.

Fig. 4. Spectra of the Fe protein of nitrogenase in zero field. Spectra (a) 77 °K, and (b) 4.2 °K are from a nitrogen-flushed sample containing 4 mg of dithionite. The sample at (c) 77 °K, is argon flushed and contains 4 mg $Na_2S_2O_4$ and ATP. The sample at (d) has ATP, but dithionite has been removed by dialysis under argon. Its concentration is lower than standard.

We cannot be sure that M1 and M2 account for the entire spectrum in neutral and mildly reducing conditions, and in fact we postulate a small amount of Fe giving rise to an additional contribution M3. We do not know its form, but we note a number of small features, such as absorptions near 2 mm/sec in Fig. 3(a) which could be in-

volved. The need for M3 will appear later. We defer discussion of the spectra of FeMo protein in strongly reducing conditions.

The main feature of the spectrum of the Fe protein in weak dithionite solution and N_2, Fig. 4(a), is a quadrupole doublet with $\delta = 0.45$ mm/sec and $\Delta E = 1.1$ mm/sec. This would be consistent with Fe^{II} or Fe^{3+}, but the latter would need some mechanism, such as spin coupling, to prevent magnetic features in the low-temperature spectrum, Fig. 4(b). (There may indeed be magnetic features in this spectrum, but they have small intensity.) We call the doublet F1 for future reference, and very tentatively assign it to Fe^{3+} spin coupled pairs. An absorption at 2.8 mm/sec, which is present in Figs. 4(a) and 4(b), was weaker in a sample flushed with argon. However, addition of ATP and removal of $Na_2S_2O_4$ both appeared to enhance it, see Figs. 4(c) and 4(d). We believe that this is one member of a high spin ferrous doublet, the other member of which lies near 0.0 mm/sec. The resultant $\delta = 1.4$ mm/sec, $\Delta E = 2.8$ mm/sec are typical of this species and nothing else. This component we name F2. It appears that conversion of Fe into the state F2 also produces absorption in the 0.6–0.7 mm/sec region. This may be part of a new doublet which we call F3. It could be that a pair of F1 Fe atoms breaks into an F2 and an F3, but this is only a speculation. A weak and diffuse absorption occurred throughout the 0.0–2.8 mm/sec range in some samples of the Fe protein at 4.2 °K.

Mossbauer spectra of the combined proteins

The Mossbauer spectra of mixtures of the proteins are shown in Fig. 5. In weak dithionite solution, Fig. 5(a), the spectrum is a simple sum of the spectra of the component proteins. A similar spectrum was observed in the presence of CO. A mixture of the proteins with ATP but no $Na_2S_2O_4$, flushed with argon, shows little evidence of the Fe protein spectrum, Fig. 5(b). We have no explanation for this and can only speculate that the Fe of the Fe protein was in such a state that its spectrum was

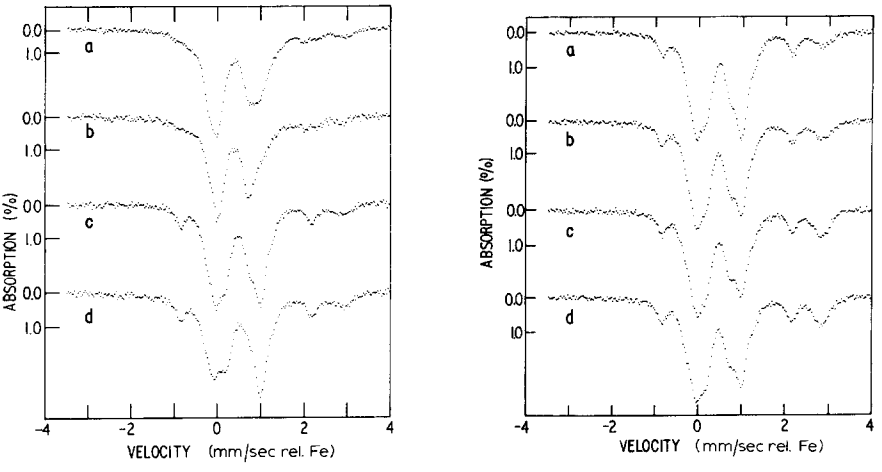

Fig. 5. Spectra of the combined argon-flushed proteins in zero field. The first three are at 77 °K with (a) dithionite but not ATP present, (b) ATP but not dithionite present, and (c) both ATP and dithionite present. The last sample exhibited spectrum (d) at 4.2 °K.

Fig. 6. Mossbauer spectra of the combined proteins, ATP, and dithionite at 77 °K in zero field with a variety of substrates. These are (a) H_2, (b) N_2, (c) acetylene, and (d) CN^-.

very broad and devoid of prominent structure. This point will require further investigation. A dithionite-free specimen flushed with N_2 yielded a spectrum similar to Fig. 5(b). As Figs. 5(c) and 5(d) indicate, a large change results when both ATP and $Na_2S_2O_4$ are present in the protein mixture. The wide magnetic spectrum M1 is removed, and new components are formed, the resultant having many of the features of the FeMo protein in strong dithionite solution. Thus it appears that the Fe protein, in the presence of ATP, brings about a change in the FeMo protein which would otherwise require a very high concentration of reductant. Before considering the details of these spectra we note their substrate dependence in the next section.

Mossbauer of nitrogenase with various substrates

The spectra of nitrogenase in the presence of various substrates is shown in Fig. 6. While there is no doubt that substrate effects are present we are not yet able to control conditions so as to make them quantitatively reproducible. With the high enzyme concentration used, saturation of the system with substrate is not easily achieved; this is particularly relevant to nitrogen because of its low solubility. Hydrogen is considered a substrate since nitrogenase catalyses an ATP- and $Na_2S_2O_4$-dependent $H_2/^2H_2O$ or $^2H_2/H_2O$ exchange[28]. Comparison of Fig. 6 with Fig. 5(c) shows that in some respects the spectrum was not altered by addition of substrate. The intensity of the lines at -0.85 and 2.2 mm/sec, henceforth called M4, did not change but the weak line at 2.8 mm/sec (F2) intensified. This was particularly marked at 77°K with acetylene or CN^- as substrate (Figs. 6(c) or 6(d)) but was also apparent with hydrogen or nitrogen (Figs. 6(a) and 6(b), respectively). It can be seen in this figure and in those that follow that the strength of the absorption at 2.8 mm/sec correlates well with an intensification of the line near 0.0 mm/sec, confirming that the left hand member of the doublet F2 lies in this region. At the same time the shape of the line near 1.0 mm/sec varies, its intensity at 1.0 mm/sec decreasing relative to its intensity at 0.8 mm/sec as the 2.8 mm/sec signal increases.

Mossbauer of nitrogenase with CO

CO competitively inhibits nitrogen fixation and addition of CO to the complete system of the two proteins, ATP and $Na_2S_2O_4$ (Fig. 7(a)) produced the same changes as observed on addition of substrate (Fig. 6(c) in particular), though the line at 2.8 mm/sec was more intense and at 77°K the doublet line at about 1 mm/sec was more altered. The decrease of signal at 1.0 mm/sec will be discussed below. At 4°K in a moderate field (5.5 kgauss) the line at 2.8 broadened out (Fig. 7(b)) confirming that the signal F2 was due to high spin ferrous. The lack of effect upon M4 suggests that it involves no unpaired spin. In Fig. 7(d) the spectrum of nitrogenase under CO in the absence of ATP is shown. The doublet M4 is not present and the spectrum is very similar to that obtained for the nitrogenase under argon (Fig. 5(a)).

Mossbauer spectra of the FeMo protein in its activated state

The spectrum of the FeMo protein in fixing mixtures is most easily seen by using Fe protein which has only the low natural abundance of ^{57}Fe. The 77°K spectra with nitrogen and CO substrates are seen in Figs. 8(a) and 8(b), respectively. These differ only in the strength of the slight absorption at 2.8 mm/sec. Similar mixtures with acetylene and with argon gave spectra which could be classed as intermediate.

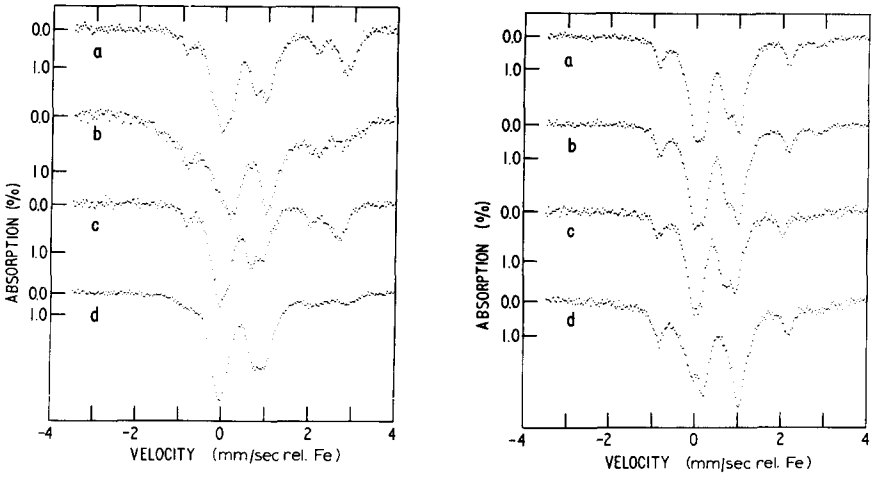

Fig. 7. The first three spectra are of the combined proteins with ATP, dithionite, and CO. Conditions are (a) 77 °K, zero field, (b) 4.2 °K, 5.5 kgauss, and (c) 195 °K, zero field. At (d), taken at 77 °K in zero field, dithionite and CO are present but not ATP.

Fig. 8. Spectra of the combined proteins in which the FeMo protein is enriched in ^{57}Fe, and the Fe of the Fe protein has only natural abundance. All samples have ATP and dithionite; conditions are (a) N_2 flushed, 77 °K, zero field, (b) CO flushed, 77 °K, zero field, (c) CO flushed, 195 °K, 390 gauss transverse field, (d) CO flushed, 4.2 °K, zero field.

Thus there appears little substrate dependence in the FeMo spectrum. The substrate effect on the Fe protein may in fact be the cause of the very small effect which was noticed. The situation is obscure, however, because a weak signal in the same region is seen in the spectrum of Fig. 2(e), where no Fe protein is present. In Figs. 8(c) and 8(d) are shown the spectra of the CO-containing mixture at other temperatures. The intensity of M4 appears constant and measurements of its area in several different spectra indicate it represents about 0.1 of the total area of the spectrum of the FeMo protein. We assume that it has a unique precursor in the resting protein, in fact the component M3 which was postulated earlier. Since the Fe pairs seem to account for almost the entire spectrum of resting FeMo protein, we assume that exclusive of M4 sites there must exist an even number of Fe atoms, making up about 0.9 of the spectrum. The assumption of 9 Fe atoms/molecule of protein would satisfy these conditions and agree well with independent estimates. The atoms would comprise four pairs in addition to the site which gives rise to M3 in resting protein and M4 in activated protein. M4 is an unusual spectrum with the large quadrupole splitting characteristic of high spin ferrous but with isomer shift too small to qualify for this assignment. Its apparent lack of unpaired spin was noted above. We tentatively identify it as low spin ferrous with extremely strong and asymmetric π bonding. This would make it analogous to oxyhaemoglobin[13] which has $\delta = 0.2$ and $\Delta E = 2.2$ mm/sec at 77 °K.

The remainder of the FeMo-activated protein spectrum seems to consist of two parts. The first, which we call M5, has $\delta = 0.6$ mm/sec, $\Delta E = 0.8$ mm/sec at 4.2 °K and little temperature dependence. It could represent Fe^{II} or coupled Fe^{3+} pairs, the isomer shift favouring the latter. An additional component, M6, has $\delta = 0.35$ mm/sec and $\Delta E = 0.7$ mm/sec at 77 °K. ΔE seems to decrease slightly at 195 °K,

and at 4.2°K M6 broadens into a diffuse magnetic spectrum indicating half integral spin. The isomer shift would favour high spin, but the diffuse appearance and variation with field at 4°K suggest spin ½. The strength of the magnetic interaction is small, suggesting the spin is delocalized and possibly that two or more sites share a single unpaired spin. The origins of M5 and M6 are therefore not clear. We note that they appear to have equal intensity. The simplest explanation would be that the action of dithionite, ATP, and Fe protein is to decouple the members of a pair, yielding one ferrous and one ferric complex. Unfortunately M5 and M6 would each be described best in terms of pairs. We might think of a molecule with four A sites and four B sites (in addition to the M4 site). In the resting state in slightly reducing conditions four A–B coupled pairs would be formed. In the activated state we would have two each of A′–A′ and B′–B′ pairs. The primes allow for changes in the electronic state while each letter designates a particular location. One can imagine geometrical arrangements which would be in keeping with this model. An example would be A sites at the corners of one face of a rhombohedron, with B sites at the corners of the opposite face. We stress the speculative nature of the present paragraph.

The spectra of the Fe protein in activated mixtures are not noticeably different from those of the isolated protein. The component F2 is especially strong in the mixture of the two proteins with dithionite, ATP, and CO. At 195°K the quadrupole splitting is seen to decrease by about 0.1 mm/sec relative to the 77°K value, Fig. 7(c) (since we cannot see the left member of F2 clearly this result is obtained from the displacement of the right hand member after allowing for the expected temperature variation of the isomer shift). At 4.2°K a field of 5.5 kgauss broadens F2 so that it seems to disappear, Fig. 7(b). This behaviour is characteristic of Fe^{2+} complex with large crystal field splittings. F2 is in fact the only completely unambiguous component we have in nitrogenase. Measurements of activated mixtures using labelled Fe protein and unlabelled FeMo protein, Fig. 9, confirm that the spectrum of the former contains the same components as in isolation. The expected F2 intensity is not developed in the CO-inhibited system, Figs. 9(b) and 9(c). In fact it is not very different from the argon-flushed system. This may be merely a manifestation of our difficulty in reliably reproducing the substrate effects, but it merits further investigation.

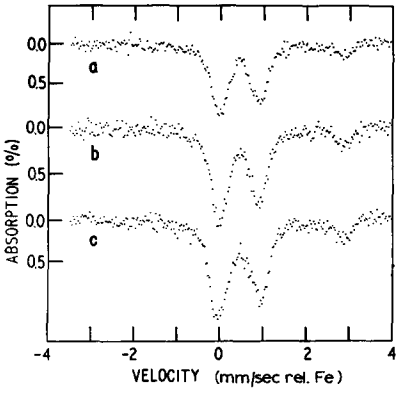

Fig. 9. Spectra of the combined proteins in which the Fe protein is enriched in ^{57}Fe and the Fe of the FeMo protein has only natural abundance. All have ATP and dithionite. Conditions are (a) argon flushed, 77°K, (b) CO flushed, 77°K, and (c) CO flushed, 4.2°K.

Effect of NO on nitrogenase

Mossbauer spectra of Fe–nitrosyl derivatives have been characterized[32], but before considering the effect of this gas on the nitrogenase spectra the effect of NO on the biochemical activity was re-examined. NO, which may act as an oxidising agent, has been reported to be a competitive inhibitor of nitrogen fixation or at least to cause reversible inhibition[9]. However, since nitrogenase is inactivated by oxygen, irreversible damage by NO might be predicted.

Acetylene reduction by nitrogenase was completely prevented by 1.5 % NO in argon. Nitrogenase *plus* $Na_2S_2O_4$, pre-incubated with 1.5 % NO for 5 min with or without ATP, then flushed 2 min under argon to remove NO had no acetylene-reducing activity, though the pH of such extracts was still 7.4 and $Na_2S_2O_4$ was present. Nitrogenase pre-incubated in a similar way under either 100 % CO or argon retained full activity. These results showed that NO caused irreversible inhibition. To determine if both proteins were equally sensitive to NO, each was pre-incubated separately with 1.5 % NO and after flushing with argon each was assayed for acetylene reduction by adding it to a system containing the other untreated protein, $Na_2S_2O_4$ and ATP. FeMo protein treated for 5 min retained full activity whereas the NO-treated Fe protein was completely inactive. Treatments of FeMo protein for periods longer than 5 min caused progressive inactivation. These results confirmed that NO rapidly inactivated Fe protein and less rapidly inactivated FeMo protein. A similar differential sensitivity of the two proteins to oxygen has been reported[19]. However, the two inhibitors showed differences since crude nitrogenase of *A. chroococcum* which was not sensitive to oxygen was irreversibly inhibited by 1.5 % NO in presence of $Na_2S_2O_4$.

Mossbauer spectra of nitrogenase in presence of NO

The spectrum observed when FeMo protein, Fe protein, ATP and $Na_2S_2O_4$ were pre-incubated for 60 sec at 15° with 0.2 % NO before freezing the sample was similar to that observed for system *plus* CN^- (Fig. 6(d)). The reduction in the 1.0 mm/sec absorption in the NO sample seemed large, however, considering the modest strength of the 2.8 mm/sec line. No evidence was seen of the characteristic Mossbauer spectrum which the unpaired NO spin is known to produce in Fe–nitrosyl complexes.

The lower NO concentration had insufficient time completely to inactivate the nitrogenase but it seems likely that Mossbauer spectroscopy of nitrogenase with NO will produce little more information than a spectrum of material inactivated by exposure to oxygen.

$^{14}CN^-$ binding to nitrogenase

The results shown in Figs. 6 and 7 suggest that neither substrate or CO react with the nitrogenase until both proteins, ATP and $Na_2S_2O_4$ are present. BUI AND MORTENSON[33], using $^{14}CN^-$ reported specific binding of this substrate to the FeMo protein alone and concluded that the substrate binding site of nitrogenase was on the FeMo protein. However, BIGGINS AND KELLY[23] have published results indicating that $^{14}CN^-$ was non specifically bound to a variety of proteins.

A further examination of $^{14}CN^-$ binding to nitrogenase was made using equilibrium dialysis under conditions described in MATERIALS AND METHODS. The results shown in Fig. 10 indicate that under CO or argon there was more radioactivity as-

sociated with nitrogenase than with dialysing buffer at equilibrium, corresponding to the non-specific binding reported previously[23]. On addition of ATP a further increase in radioactivity associated with nitrogenase was observed under argon, but not under CO (which inhibited CN^- reduction at higher temperatures). The probable explanation of these observations (as the Mossbauer spectra suggest) is that only in the presence of both proteins, $Na_2S_2O_4$ and ATP is CN^- bound specifically.

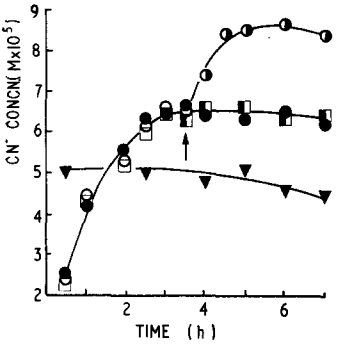

Fig. 10. $^{14}CN^-$ associated with nitrogenase. Three separate dialyses were set up using procedures described in MATERIALS AND METHODS. ▼—▼, radioactivity associated with buffer (average of three); ●—●, radioactivity associated with nitrogenase under argon, no ATP added. Radioactivity associated with nitrogenase under argon: ○—○, before; ◑—◑, after ATP addition. Radioactivity associated with nitrogenase under CO: □—□, before; and ◧—◧, after ATP addition marked by ↑.

DISCUSSION

Broadly speaking, in its biological role the nitrogenase serves to collect electrons from the donor system, to bind the substrate nitrogen and either to insert electrons while permitting the complex to combine with protons of the solution or directly to effect substrate reduction by protein hydride attack. With respect to the present study the following questions are relevant: (1) Does the Fe clearly participate in the processes just described? (2) Can we determine the nature of the environment of the Fe atoms? (3) Can particular Fe atoms be related to particular parts of the fixation process? The Mossbauer spectra presented above contain a wealth of information, not all of it completely understood. Some of the results we believe to be both significant and well established, and we shall list these before proceeding to those whose interpretation is less certain.

The most outstanding feature of the Mossbauer spectra of nitrogenase is the appearance of certain spectral features only under conditions which are known to be necessary and sufficient for fixation. Only when both proteins, ATP and $Na_2S_2O_4$ are all present does the removal of the broad magnetic spectrum and the appearance of M4 and F2, low and high spin ferrous irons, occur. Thus, in answer to (1) there seems little doubt that Fe is a participant in reduction.

It is virtually impossible from Mossbauer data alone to characterize completely the environment of the resonant nucleus, and many of the spectral components of the nitrogenase admit a variety of interpretations. The features of the magnetic spectrum M1 and its variation with reductant concentration indicate very strongly

that the electron spins on different Fe atoms interact, and hence that these atoms are arranged in pairs or larger multiples with small separations, probably not more distant than second neighbours. The situation is analogous to that of ferredoxin, but differences between their Mossbauer spectra indicate that it would be incorrect to regard the FeMo protein as a type of ferredoxin. Thus in answer to (2) we have the firm and important conclusion that some of the Fe occurs in pairs or larger multiples.

The spectra exhibited in the presence and absence of a variety of substrates and the inhibitor CO (Figs. 6 and 7) appear to have identical components, and differ only in the relative strength of these components. Because the Mossbauer spectrum is sensitive to the Fe ligands, this indicates that at any instant no significant fraction of the Fe is bound to substrate or inhibitor. This is related to both (2) and (3) above. In the following paragraphs we discuss interpretations which we must regard as less firm than the preceding.

Although the relevant component cannot be identified in the Mossbauer spectra, Fe could still be involved in substrate binding if the concentration of the complex were low. If we are correct in our interpretation that F1 from Fe protein is Fe^{3+} and F2 is Fe^{2+}, the substrate is causing an increase in reduction. In the absence of substrate, interactions of electrons from FeMo protein with ATP and Fe protein may produce a reduced site at which hydrogen evolution occurs and the presence of an electrophilic substrate might then result in transfer of this reducing power to another site involved in substrate reduction. Some correlation of the strength of the F2 component with the rate of reduction suggests that F2 is related to the binding affinity. Also F2 was greatest in the presence of CO (Fig. 7) which, though it inhibits nitrogen fixation, does not affect the rate of ATP-dependent hydrogen evolution which nitrogenase catalyses in the absence of added substrate. However, since F2 was weak under argon when hydrogen would be evolved at the same rate as under CO, this line probably does not represent the hydrogen producing site of nitrogenase (ref. 9). Since F2 was most intense when not merely both proteins, ATP and $Na_2S_2O_4$ were present but also substrate or CO and because it was apparently derived from Fe protein (Fig. 9) the obvious inference is that Fe protein contributes to the substrate binding site.

Both the Mossbauer data and the experiment with equilibrium dialysis indicate that substrate or inhibitor only bind in the presence of all the other components. Thus Fig. 7(d) shows that no F2 line was present with CO, FeMo protein, $Na_2S_2O_4$ and Fe protein in the absence of ATP. Consequently either the substrate binding site is not accessible in the Fe protein until after stereochemical changes have occurred involving all the other components or the active site is only formed in the active complex of both proteins after ATP and $Na_2S_2O_4$ reactions.

Although we believe the F2 effect is real and significant it would be imprudent not to note that a signal similar to F2 has been reported in $Na_2S_2O_4$-reduced ferredoxin and was later found to be associated with denatured protein[34]. Furthermore, we have been able to produce an F2-like spectrum in FeMo protein by dialysing away the $Na_2S_2O_4$ under argon, stirring 7 min in air, and then adding a few mg/ml of $Na_2S_2O_4$.

The variation in intensity of the M1 spectrum with $Na_2S_2O_4$ concentration (Figs. 2, 3) and its dependence on the number of electrons on the Fe strongly suggest that the postulated pairs in the FeMo protein are involved with collection and/or storage of electrons. Since six electrons are necessary for nitrogen reduction, the

presence of either three or six pairs would be particularly suggestive. If, as we have assumed, M4 represents one Fe atom, and has a characteristic precursor in the resting state, then the most likely number of Fe atoms remaining is eight. These may comprise four equivalent pairs, but we have never seen the M1 component larger than about 5/6 of the total spectrum of resting FeMo protein. Thus it may be that only three pairs are potential electron collectors which supply in parallel the electrons necessary for reduction. A more thorough investigation of the dependence on reductant concentration is obviously in order.

These studies indicate areas where further application of Mossbauer spectroscopy could usefully be made. The effect of ATP, substrate or protein concentration and rate of freezing on the spectrum may enable exactly reproducible spectra to be obtained. More detailed examination of the spectrum of each protein alone under controlled oxidising and reducing conditions may help to clarify the origin and significance of certain lines, in particular, F2. The use of a natural reductant for nitrogenase is necessary to confirm that all the spectral components so far observed are significant and not merely a consequence of using the artificial electron donor $Na_2S_2O_4$. Since natural reductants are also Fe-containing proteins (*e.g.* ferredoxin) a very complicated Mossbauer spectrum would result. Other possibilities for study include Mossbauer spectroscopy of nitrogenase from other nitrogen-fixing bacteria and use of combinations of FeMo protein from one organism with Fe protein from another in combinations that do not work well together (*cf.* ref. 19).

In conclusion this Mossbauer spectroscopy clearly shows the considerable involvement of Fe in the enzymic functioning of nitrogenase and emphasises the special value of Mossbauer spectroscopy, particularly when used in conjunction with more standard enzyme assays, in probing the role of Fe in biological systems.

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