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Mössbauer Spectroscopy of the Nitrogenase Proteins from *Klebsiella pneumoniae*

STRUCTURAL ASSIGNMENTS AND MECHANISTIC CONCLUSIONS

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The Mo–Fe protein and the Fe protein which together constitute the nitrogenase of *Klebsiella pneumoniae* were prepared from bacteria grown in ^{57}Fe-enriched medium. The Mössbauer spectrum of the Mo–Fe protein, as isolated in the presence of $Na_2S_2O_4$, showed that the protein contained three iron species, called M4, M5 and M6. The area of the spectrum associated with species M4, with $\delta = 0.65$ mm/s and $\Delta E = 3.05$ mm/s at 4.2°K, corresponded to two iron atoms/molecule of protein and it is interpreted as being due to a high-spin ferrous, spin-coupled pair of iron atoms. The iron atoms of species M4 may be involved in the quaternary structure of the protein. Species M5, with $\delta = 0.61$ mm/s and $\Delta E = 0.83$ mm/s at 77°K, corresponded to eight iron atoms/molecule of protein and is interpreted as being due to Fe_4S_4 or Fe_2S_2 low-spin ferrous iron clusters. Species M6, with $\delta = 0.37$ mm/s and $\Delta E = 0.71$ mm/s at 77°K, also corresponded to eight iron atoms/molecule of protein and, at 4.2°K, became a broad shallow absorption, characteristic of magnetic hyperfine interaction. Oxidation of the Mo–Fe protein with the redox dye Lauth's Violet did not affect the activity of the protein but changed species M4, M5 and M6 into the species M1 ($\delta = 0.37$ mm/s, $\Delta E = 0.75$ mm/s at 77°K, broad magnetic component at 4.2°K) and M2 ($\delta = 0.35$ mm/s, $\Delta E = 0.9$ mm/s at 4.2°K). In the presence of the Fe protein, $Na_2S_2O_4$, ATP and Mg^{2+}, the M6 component of the Mo–Fe protein was replaced by species M7 with $\delta = 0.46$ mm/s, $\Delta E = 1.04$ mm/s at 4.2°K. The change in Mössbauer parameters associated with the M6 → M7 transformation was very similar to the change observed on reduction of the high-potential Fe protein from *Chromatium vinosum*. In contrast, $Na_2S_2O_4$-reduced Fe protein contained only one type of iron cluster (F4). Species F4 had $\delta = 0.50$ mm/s, $\Delta E = 0.9$ mm/s at 195°K, and at 4.2°K broadened in a manner characteristic of a magnetic hyperfine interaction, associated with half-integral spin, equally distributed over all four atoms of the Fe protein. The Mössbauer spectra of the Mo–Fe and the Fe protein under argon were unaffected by the reducible substrates N_2 and C_2H_2 and the inhibitor CO in the presence of ATP, Mg^{2+} and $Na_2S_2O_4$. A number of Mössbauer spectral species associated with inactivated Mo–Fe and Fe proteins are described and discussed.

Nitrogenase from a number of micro-organisms has been separated into two proteins, the larger containing molybdenum, iron and acid-labile sulphur, and the other iron and acid-labile sulphur. Both proteins are rapidly inactivated by exposure to O_2. Neither protein has any catalytic activity alone, but when they are recombined and added to ATP, Mg^{2+} and a reducing agent will reduce N_2 to NH_3, H^+ to H_2, and also a number of other triple-bonded substrates (Burris, 1971).

Klebsiella pneumoniae fixes N_2 under anaerobic conditions. The Mössbauer spectroscopy of nitrogenase from this organism was the subject of an earlier study (Kelly & Lang, 1970), but since that time the constituent proteins have been further purified (Eady *et al.*, 1972), resulting in complete separation of the two proteins, with specific activities about five times higher than those of the earlier preparations and higher metal contents. Eady *et al.* (1972) found that the Mo–Fe protein of *K. pneumoniae*, Kp1 protein, contained one Mo, 17–18 Fe and 17 labile S atoms in mol.wt. 218000. The Fe protein, Kp2 protein, contained four iron and four labile S atoms in mol.wt. 66800.

The improved purity, together with much closer control of the oxidation state of the proteins, resulted in the changes in the Mössbauer spectra and their interpretation presented here.

Recent e.p.r.* studies on nitrogenase from

*Abbreviation: e.p.r. = electron paramagnetic resonance.

Klebsiella pneumoniae (Smith *et al.*, 1972, 1973), *Clostridium pasteurianum* (Orme-Johnson *et al.*, 1972; Mortenson *et al.*, 1973) and *Azotobacter vinelandii* (Orme-Johnson *et al.*, 1972) have shown that the Fe protein, after interaction with ATP and Mg^{2+}, acts as a very specific electron carrier to the Mo–Fe protein. The e.p.r. evidence involved changes in the intensities of the e.p.r. signals exhibited by both proteins in the presence of $Na_2S_2O_4$, ATP and Mg^{2+}. These signals are associated with the iron atoms in both proteins.

Smith *et al.* (1972, 1973) found that ATP-activated electron transfer from Kp2 protein to Kp1 protein occurred much faster than the turnover time of the enzyme and they postulated that it resulted in a 'super-reduced' form of Kp1 protein, which then reduced the substrate in what was probably the rate-determining step of the enzymic reaction. However, Zumft *et al.* (1972) and Mortenson *et al.* (1973) have favoured a reaction scheme in which the Mo–Fe protein cycled between the normal $Na_2S_2O_4$-reduced form of the protein and an oxidized form, with rate-limiting electron transfer from the Fe protein to the Mo–Fe protein. Smith *et al.* (1972, 1973) showed that this step was not rate-limiting but concluded that the e.p.r. evidence alone did not completely exclude variants of the scheme of Mortenson *et al.* (1973).

The Mössbauer data presented here provide further evidence in support of the mechanism proposed by Smith *et al.* (1972, 1973).

Materials and Methods

^{57}Fe

A sample (100mg) of ^{57}Fe-enriched $FeSO_4$ was obtained from the United Kingdom Atomic Energy Commission, Harwell. The sample, which had a mass analysis 0.23% ^{54}Fe, 12.19% ^{56}Fe, 87.44% ^{57}Fe and 0.13% ^{58}Fe, was dissolved in 100ml of dilute HNO_3 by gentle warming.

Growth of K. pneumoniae cells on ^{57}Fe-containing media

The methods used for removing Fe from glassware and media, and for growth and harvesting of cells, were those described by Kelly & Lang (1970), with the following modifications.

(1) For washing the glassware 50% (v/v) HNO_3 was used in place of conc. H_2SO_4.

(2) The concentrated phosphate buffer solution was autoclaved with chromatographic-quality Al_2O_3 (100g/l) as described by Donald *et al.* (1952). This treatment was simpler and more efficient at removing iron than washing with 8-hydroxyquinoline in chloroform solution. The Al_2O_3 was removed by filtration and centrifugation.

(3) Despite (2) the residual iron concentration in the buffer solution was still high enough to give a final concentration of approx. 0.15mg of Fe/l, i.e. 15% of the ^{57}Fe added. Therefore only 25% of the normal buffer concentration was used in the 20-litre pot cultures (normal buffer concentrations were used in the inocula). The pH of the 20-litre cultures was maintained at 6.5 by automatic addition of 5M-KOH solution.

Purification and preparation of nitrogenase proteins

The nitrogenase proteins were purified by the methods of Eady *et al.* (1972). The specific activity of Kp1 protein preparations varied from 1000 to 1400nmol of C_2H_4 produced/min per mg of Kp1 protein and that of Kp2 protein preparations varied from 700 to 1000nmol of C_2H_4 produced/min per mg of Kp2 protein. The protein preparations were homogeneous by the criteria of polyacrylamide gel electrophoresis (Hedrick & Smith, 1968) and sodium dodecyl sulphate gel electrophoresis (Weber & Osborn, 1969).

After purification the Kp1 protein was concentrated by membrane fiiltration at 0–5°C. Kp2 protein was concentrated first by absorption on to DE32 DEAE-cellulose and elution with 90mM-$MgCl_2$ in 25mM-Tris–HCl buffer, pH7.4 containing 100mg of dithiothreitol/l and 1mM-$Na_2S_2O_4$, followed by membrane filtration.

Preparation of Mössbauer samples

Mössbauer spectra were run on samples contained in flat-faced polythene holders (internal dimensions 3mm×14mm×20mm). These were placed in a cylindrical vessel (2.5cm×15cm) fitted, near the top, with a side arm with which it was attached to a vacuum/flushing line. The vessel and holders were degassed to below 0.67Pa (5×10^{-3}mmHg), and flushed three times with argon. The holders were then filled with the protein solutions under a constant stream of argon, plunged quickly into liquid N_2 and frozen.

Samples of individual proteins were typically 50mg of Kp1 protein/ml (23μM) in 25mM-Tris–HCl, pH7.4, containing 100mg of dithiothreitol/l and 1mM-$Na_2S_2O_4$, or 30mg of Kp2 protein/ml (45μM) in the same buffer also containing 50mM-$MgCl_2$. Mixtures of proteins were approx. 12.5μM in both Kp1 and Kp2 proteins.

ATP was always added with Mg^{2+} and a regenerating system of creatine kinase and creatine phosphate. This is referred to as the ATP system. Typically 0.1ml of this system containing 8μmol of ATP, 15μmol of creatine phosphate, 20μmol of $MgCl_2$, 40μmol of Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid]–NaOH buffer, pH7.8,

and 0.2mg of creatine kinase, was added to 0.6ml of protein solution.

To determine the effect of substrates on a mixture of the two proteins, they were incubated for 10min in a small bottle, sealed with a Subaseal closure, under Ar, N_2, C_2H_2+Ar (50:50) or CO+Ar (50:50). Each sample was then transferred anaerobically to a degassed holder, which already held 0.1ml of the ATP system containing 20μmol of $Na_2S_2O_4$. The solutions were mixed and then frozen in liquid N_2 within 1min.

The Mössbauer spectrometer was that described by Kelly & Lang (1970). Sources of gases and chemicals are given by Eady *et al.* (1972).

In the Mössbauer data isomer shifts, δ, and the horizontal axes in the figures, are relative to metal iron as standard.

Results and Discussion

Mössbauer spectra of Kp1 protein

Comparison with the data of Kelly & Lang (1970). Kelly & Lang (1970) observed two major spectral species in solutions of Kp1 protein as prepared in the presence of weak $Na_2S_2O_4$: M1 ($\delta = 0.37$mm/s; $\Delta E = 0.75$mm/s at 77°K; wide paramagnetic spectrum at 4.2°K) and M2 ($\delta = 0.35$mm/s; $\Delta E = 0.4$mm/s at 4.2°K). After incubation with very concentrated $Na_2S_2O_4$ (20mg/ml) or with Kp2 protein, ATP, Mg^{2+} and an ATP-regenerating system, the species M1 and M2 were transformed into species M4 ($\delta = 0.65$mm/s; $\Delta E = 3.05$mm/s at 4.2°K), M5 ($\delta = 0.6$mm/s; $\Delta E = 0.8$mm/s at 4.2°K) and M6 ($\delta = 0.35$mm/s; $\Delta E = 0.7$mm/s at 77°K; indistinct at 4.2°K). These authors also postulated the presence of species M3, a precursor of species M4, in the weak $Na_2S_2O_4$ solutions. To avoid confusion and to enable easier comparison with the data of Kelly & Lang (1970), we shall use their nomenclature for the various spectral species wherever possible.

Fig. 1 shows the effect of progressive exposure of Kp1 protein to O_2. Spectrum (*a*) of Fig. 1 is typical of the spectra obtained for Kp1 protein as normally prepared in the presence of 1mM-$Na_2S_2O_4$, although the $Na_2S_2O_4$ had been removed from the sample by anaerobic chromatography on a Sephadex G-25 column.

The series of spectra in Fig. 1 demonstrates that exposure to air gradually changed all the iron in Kp1 protein, and that the intensity of a broad magnetic species (M1 of Kelly & Lang, 1970) passed through a maximum and then decreased as oxidation proceeded. This oxidation sequence differs from that of Kelly & Lang (1970). Our spectrum of the $Na_2S_2O_4$-reduced protein corresponds to theirs after incubation with 20mg of $Na_2S_2O_4$/ml and our spectrum after partial oxidation of the protein corresponds approximately

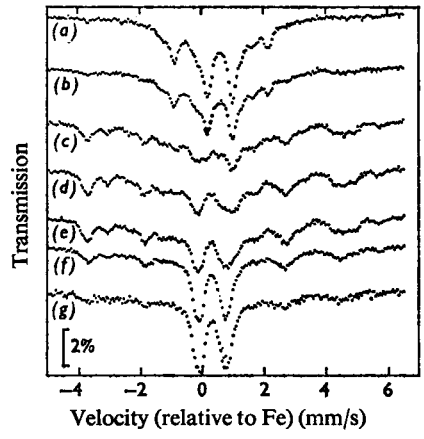

Fig. 1. *Effect of progressive aeration on the Mössbauer spectrum of Kp1 protein*

(*a*) Kp1 protein as isolated but after removal of $Na_2S_2O_4$ on an anaerobic Sephadex G-25 column. (*b*) Sample (*a*) thawed, stirred in air for 30s and refrozen. (*c*)–(*g*) Sample (*a*) was treated as in (*b*) but with 90, 150, 210, 490 or 1390s total aeration respectively. All spectra were measured at 4.2°K in a 550G magnetic field perpendicular to the γ-ray beam.

to their protein spectrum in weak $Na_2S_2O_4$ solutions.

The handling techniques that we used for the preparation of Mössbauer samples (see the Materials and Methods section) exclude O_2 much more rigorously than those described by Kelly & Lang (1970). We have observed that the reduction of oxidized Kp1 protein by $Na_2S_2O_4$ is slow, and this observation has been confirmed by visible spectroscopy and e.p.r. studies (Smith *et al.*, 1972).

We must therefore conclude that the spectra reported by Kelly & Lang (1970) for Kp1 protein in the presence of dilute $Na_2S_2O_4$ represent samples that were inadvertently oxidized during preparation and did not have enough time, before freezing, to react with the $Na_2S_2O_4$ added at that time.

With this single assumption the two sets of data on Kp1 protein are compatible despite the differences in specific activity and iron content of the proteins used.

$Na_2S_2O_4$-reduced Kp1 protein. The spectra of the $Na_2S_2O_4$-reduced Kp1 protein samples (Figs. 2*a* and 2*b*) at 195°K and 77°K appear to consist mainly of three partially overlapping quadrupole pairs of absorptions, in addition to some small contributions which are seen only as distortions of the main lines. These three major species correspond to species M4, M5 and M6 of Kelly & Lang (1970).

Although computer fitting under these conditions is hazardous we consider that, in moderation, it can be an advance over visual inspection of the spectra. We therefore submitted the spectra to a least-squares fitting programme, assuming three quadrupole pairs and allowing the isomer shift, quadrupole splitting, line width and intensity of each to vary. This provided a fairly consistent picture, as shown in Table 1. The temperature-dependence of isomer shift is as one would expect from the second-order Doppler effect, and the presence of a null or small decrease of quadrupole splitting with increased temperature is typical of complexes with no low-lying electronic orbital excitations. The intensities are given as numbers of atoms out of a total of 18 iron atoms in Kp1 protein, the assumption being made that their recoil-less fractions are all equal. In the spectra of Kp1 protein measured at 4.2°K (Figs. 2*c*, 2*d* and 2*e*) the M6 component is replaced by a broad and shallow absorption characteristic of magnetic hyperfine interaction. Its change on application of a small field and sensitivity to field direction suggests that the iron atoms of species M6 contain some half-integral unpaired electron spin. In the absence of a model for the magnetic part, a least-squares fit was not possible. Therefore to estimate the strength of species M4 and M5 in Fig. 2(*c*), we have tried to subtract from it their expected contributions, based on the fit at 77°K, by varying their intensity until they appear to be eliminated. This very rough procedure indicated that species M4 represented the same fraction of the iron at 4.2°K as it did at 77°K, whereas species M5 was either the same or relatively slightly weaker at the lower temperature. We therefore attribute the magnetic background entirely to species M6. Protein Kp1 is an iron–sulphur protein and we can reasonably expect it to have some interacting clusters with two or four iron atoms. The intensities of components M4, M5 and M6 correspond closely to two, eight and eight iron atoms/molecule respectively, suggesting that such clusters are present.

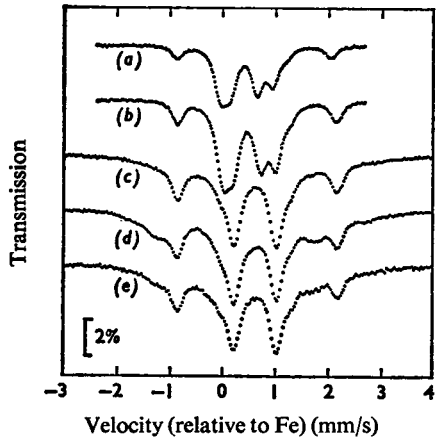

Fig. 2. *Mössbauer spectra of Kp1 protein as isolated in the presence of* $Na_2S_2O_4$

(*a*) 195°K; (*b*) 77°K; (*c*) 4.2°K, zero magnetic field; (*d*) 4.2°K, 100 G magnetic field parallel to the γ-ray beam; (*e*) 4.2°K, 55.0 G magnetic field perpendicular to the γ-ray beam.

The component M4 has a quadrupole splitting typical of high-spin ferrous materials, but its isomer shift is unusually low for this class. However, both parameters ($\delta = 0.60$ mm/s; $\Delta E = 2.90$ mm/s at 195°K) reasonably approximate to the values found for a component of reduced spinach ferredoxin ($\delta = 0.56$ mm/s; $\Delta E = 2.65$ mm/s at 195°K) (Rao *et al.*, 1971; Dunham *et al.*, 1971), which is well characterized as a high-spin ferrous iron atom. In ferredoxin this iron atom is magnetically coupled to a high-spin ferric iron atom, and was shown by the above workers to have strong magnetic features in its helium-temperature Mössbauer spectra. The absence of magnetic features in the low-temperature spectra of species M4 in both zero and small magnetic fields indicates that it is not coupled to a half-integral spin system. As noted by Kelly & Lang (1970), and more recently verified by us, measurements of species M4 in high field reveal an absence of unpaired integral spin as well. This ruled

Table 1. *Least-squares resolution of spectra of Kp1 protein into its components*

Isomer shift, δ, is relative to metal iron. Number of atoms is out of an assumed total of 18 in the molecule. The data, at each temperature, are given for three different Kp1 protein samples.

	Species .. M4			M5			M6		
Temperature	δ (mm/s)	ΔE (mm/s)	Atoms	δ (mm/s)	ΔE (mm/s)	Atoms	δ (mm/s)	ΔE (mm/s)	Atoms
195°K	0.60	2.93	2.2	0.56	0.83	7.3	0.32	0.69	8.4
	0.60	2.92	1.9	0.56	0.83	7.8	0.32	0.68	8.3
	0.60	2.92	2.2	0.56	0.84	8.0	0.32	0.70	7.8
77°K	0.67	3.02	2.3	0.61	0.82	8.2	0.37	0.70	7.5
	0.66	3.02	2.3	0.61	0.83	8.1	0.37	0.70	7.6
	0.66	3.02	2.4	0.61	0.84	8.9	0.37	0.72	6.7

out a high-spin ferrous assignment in the interpretation of Kelly & Lang (1970), where the intensity of species M4 was thought to represent one iron atom of a total of nine or ten in a molecule of Kp1 protein. Since present indications are that this component represents two iron atoms in a molecule containing 18, there exists the attractive possibility of interpreting species M4 as a pair of high-spin ferrous iron atoms spin-coupled so as to have no net unpaired spin. Each molecule of Kp1 protein consists of two pairs of apparently identical subunits with molecular weights of approx. 50000 and 60000 respectively (Eady *et al.*, 1972). On the basis of symmetry, one might expect each iron atom of species M4 to be bound to a different polypeptide chain, and that two identical polypeptides are geometrically organized within the molecule of Kp1 protein so that these two iron atoms can be spin-coupled, presumably through sulphide bridges. If this symmetry-based argument is correct then the two iron atoms of species M4 may be important in defining the quaternary structure of Kp1 protein.

Apart from improved accuracy in the quadrupole-splitting and isomer-shift data, we have no information about species M5 beyond that reported by Kelly & Lang (1970). We favour their alternative assignment of low-spin ferrous iron for this component for two reasons. First, this is a reduced system, so ferrous is the more likely form, and secondly, the known high-spin ferric coupled pairs in iron–sulphur proteins have isomer shifts typically in the region of 0.25mm/s at 77°K and lower (Dunham *et al.*, 1971), in contrast with $\delta = 0.61$ mm/s for species M5.

Species M5 corresponds to eight iron atoms/molecule of Kp1 protein, which on oxidation appear to be spin-coupled (see below), and therefore we consider that it probably consists of two Fe_4S_4 or four Fe_2S_2 structures symmetrically distributed between the subunits of Kp1 protein. However, these structures must be different from those in the ferredoxins, which contain only high-spin iron. We will reserve further discussion of the nature of species M6 until the section on protein Kp1–Kp2 interactions.

Oxidation of Kp1 protein. We now turn our attention to the oxidized Kp1 protein species shown in Figs. 1 and 3. Kp1 protein oxidized by the redox dye Lauth's Violet (Fig. 3) retained full enzymic activity, but the species M4, M5 and M6 had disappeared and were replaced by species M1 and M2 of Kelly & Lang (1970).

The spectra shown in Fig. 3 correspond approximately to those obtained after 490s exposure to air (Fig. 1), but the lines are rather sharper and better defined. Progressive oxidation with $K_3Fe(CN)_6$ produced similar changes to air oxidation. Thus all oxidations seem to involve a similar series of changes, although the relative fuzziness of the species M1 line-shapes in air-oxidized Kp1 protein relative to Lauths's Violet-oxidized Kp1 protein may indicate O_2 damage, perhaps associated with a structural change of the protein, which, although not directly involving the bonding of the iron atoms, nevertheless affects their environment.

Our analysis of these spectra of the oxidized protein does not differ from that of Kelly & Lang (1970). Species M1 ($\delta = 0.37$mm/s; $\Delta E = 0.75$mm/s at 77°K) is a complex mixture of magnetic species, which, since further oxidation decreases its intensity, must be due to coupled pairs or higher multiples of iron atoms. Species M2 ($\delta = 0.35$mm/s; $\Delta E = 0.9$mm/s), an oxidized species, with an isomer shift corresponding to that of high-spin ferric iron, but without magnetic character, is also probably due to spin-coupled pairs or higher multiples of iron atoms.

We were not able to distinguish any discontinuities in the oxidation series (Fig. 1). Species M4 and M5 seemed to disappear together (species M6 cannot easily be distinguished at 4.2°K), and their disappearance coincided with the appearance of all parts of the M1 spectrum, although this is clearly due to a mixture of species. Similarly, on further oxidation, there was an apparently simultaneous decrease in intensity of all parts of the M1 spectrum.

O_2-damaged Kp1 protein. Figs. 4(*a*) and 4(*b*) show the spectra of a Kp1 protein sample retaining only 2–3% of its specific activity after prolonged exposure to air at 4°C. The major feature is a doublet ($\delta = 0.40$mm/s; $\Delta E = 0.75$mm/s at 77°K), and in addition there is evidence of a broad magnetic species at 4.2°K. This species was much more apparent when the protein had been exposed to O_2 at 30°C (Figs. 4*c*

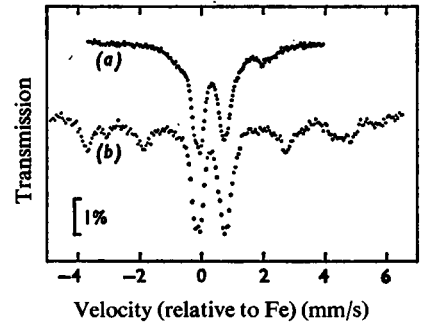

Fig. 3. *Mössbauer spectra of undamaged Kp1 protein oxidized by Lauth's Violet*

(*a*) 77°K; (*b*) 4.2°K, 550G magnetic field perpendicular to the γ-ray beam.

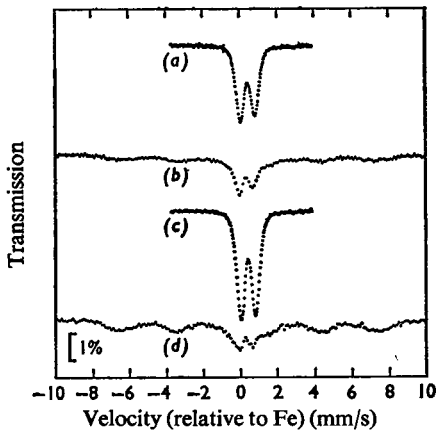

Fig. 4. *Mössbauer spectra of Kp1 protein after inactivation by exposure to air*

The first two spectra are of a sample from which $Na_2S_2O_4$ had been removed before exposure to air for 16h at 4°C: (*a*) 77°K; (*b*) 4.2°K. Spectra (*c*) (77°K) and (*d*) (4.2°K) are of the sample used for the experiment in Fig. 1, after a further 2½h exposure to air in a shaking water bath at 30°C. Some precipitation of the protein occurred towards the end of this treatment. The 4.2°K spectra (*b*) and (*d*) were measured in a 550G magnetic field perpendicular to the γ-ray beam.

and 4*d*). At this temperature some of the protein had precipitated. These two species are different from any others that we have observed in Kp1 protein, although a similar spectrum to Fig. 4(*d*), but with different temperature-dependence, was reported by Moshkovskii *et al.* (1971) for the freeze-dried whole cells of *Ectothiorhodospira shaposhnikovii* grown under N_2-fixing conditions.

Our interpretation of the above data is that the spectra arise from super-paramagnetic clusters of ferric oxide or oxy-hydroxide, which are either free of the protein or are non-specifically bound to it. It is typical of such systems that the spectrum at relatively high temperature (e.g. 77°K) is a quadrupole doublet. As the temperature is lowered this decreases in intensity, while a magnetic spectrum becomes more intense. At high temperature the spin of a cluster changes direction rapidly. The spins on individual ions are tightly coupled to it, and their resultant rapid relaxation is sufficiently fast to suppress observable magnetic hyperfine interaction. As the temperature is lowered the net spin and hence the constituent spins relax more slowly and a magnetic hyperfine structure appears in the Mössbauer spectrum. The growth of the magnetic spectrum at the expense of the simple quadrupole doublet is spread over a range of temperature which depends on the distribution of particle sizes, the larger particles tending to relax more slowly at a given temperature.

The isomer shift and the quadrupole splitting observed in Fig. 4 correspond closely to those of ferritin (Boas & Window, 1966), which at 77°K has $\delta = 0.38 \pm 0.02$mm/s, $\Delta E = 0.74 \pm 0.04$mm/s. This material consists of granules of $(FeO \cdot OH)_8(FeO \cdot OPO_3H_2)$ embedded in protein. The effective field deduced from Fig. 4, 440kG, is nearer to that of γ-FeO·OH, 460kG (Johnson, 1969), than to that of ferritin, 493±10kG (Boas & Window, 1966). Boas & Window (1966), using ferritin, whose micelles are known to be about 5.5nm in diameter, observed a spectrum at 29°K similar to Fig. 4(*d*). Since the relaxation rate is expected to depend on the ratio of particle volume to temperature we can estimate that in the present case the particle diameter is about 3–4nm (assuming we have the same anisotropy energy per unit volume). Of the two damaged Kp1 protein samples, the particle size is apparently larger in the one in which protein has precipitated. In each case it is unlikely that the observed spectrum has any relevance to the structure of nitrogenase. However, these observations do indicate that one cause of the irreversibility of O_2 damage of Kp1 protein is the loss of iron.

Mössbauer spectra of Kp2 protein

$Na_2S_2O_4$-reduced Kp2 protein. The Mössbauer spectra of reduced Kp2 protein at 195°K, 77°K and 4.2°K are shown in Fig. 5.

The 195°K spectrum has the appearance of a symmetrical doublet with $\delta = 0.47$mm/s, $\Delta E = 0.95$mm/s, but at 77°K some asymmetry is apparent; in particular a shoulder has appeared at about 1.3 mm/s. At 4.2°K the original doublet has broadened into a multiplet, indicating magnetic character. These spectra, which we shall attribute to a species F4, are totally unlike those obtained by Kelly & Lang (1970). The 195°K spectrum suggests that the iron nuclei are equivalent or at least remarkably similar in their interactions with the local electronic charge distributions. At the lower temperatures magnetic interactions are present and in the absence of a specific model the question of homogeneity is unresolved. However, the 4.2°K spectra and the effect of the orientation of the external magnetic field on them are consistent with unpaired half-integral spin distributed over all four iron atoms with a small internal field.

Oxidized and O_2-damaged Kp2 protein. We have been unable, as yet, to oxidize Kp2 protein without damage, but Figs. 6(*a*) and 6(*b*) show the 77°K and 4.2°K spectra of Kp2 protein oxidized by excess of 2,6-dichlorophenolindophenol. This oxidation resulted in the loss of approximately half of the activity of the protein; the spectra are probably due to a

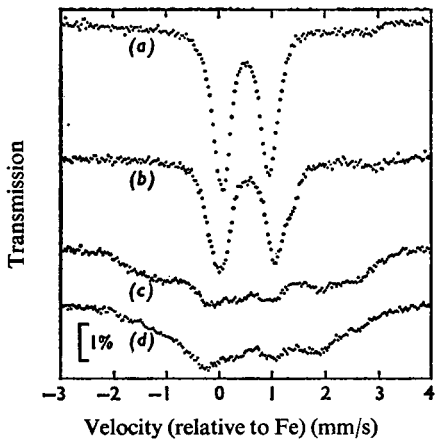

Fig. 5. *Mössbauer spectra of Kp2 protein as prepared in the presence of* $Na_2S_2O_4$

(*a*) 195°K; (*b*) 77°K; (*c*) 4.2°K, 100G magnetic field parallel to the γ-ray beam; (*d*) as (*c*) with 550G magnetic field perpendicular to the γ-ray beam.

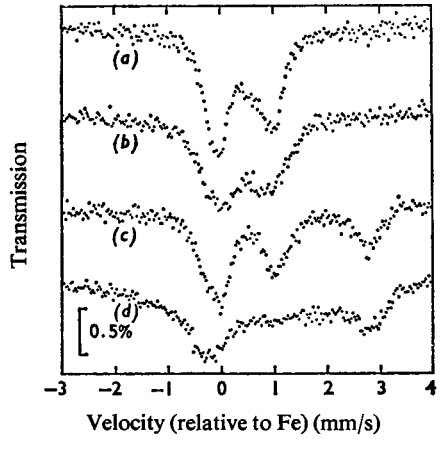

Fig. 6. *Mössbauer spectra of Kp2 protein oxidized by 2,6-dichlorophenol-indophenol*

(*a*) 77°K; (*b*) 4.2°K, and then reduced by $Na_2S_2O_4$; (*c*) 77°K; (*d*) 4.2°K. The 4.2°K spectra were measured in a 550G magnetic field perpendicular to the γ-ray beam.

mixture of species with the average parameters $\delta = 0.47$mm/s, $\Delta E = 1.05$mm/s at 77°K. The original reduced Kp2 protein (F4) had $\delta = 0.52$mm/s, $\Delta E = 1.05$mm/s at 77°K, the observed change in isomer shift being consistent with an oxidation.

Reduction of the oxidized protein with $Na_2S_2O_4$ resulted in the spectra shown in Figs. 6(*c*) and 6(*d*). These consist partially of the spectral features of species F4 and partially of a doublet with $\delta = 1.4$mm/s, $\Delta E = 2.8$mm/s at 77°K. We have also observed this latter species in reduced O_2-damaged samples of both Kp2 and Kp1 proteins. Kelly & Lang (1970) called this species F2, and reported that its intensity varied in the presence of different reducible substrates, while noting that its parameters were very similar to those of denatured ferredoxin. We now conclude that species F2 is due to denatured Kp2 protein and that the substrate effects on its intensity do not reflect the binding of reducible substrates to active nitrogenase.

The major Kp2 protein species observed by Kelly & Lang (1970), F1, had $\delta = 0.45$mm/s, $\Delta E = 1.1$mm/s at 77°K. We only observed this species in samples that had been damaged by O_2 and consequently had lost at least two-thirds of their original activity. Subsequent incubation with $Na_2S_2O_4$ failed to convert species F1 to F4. These observations forced us again to the conclusion that the preparation of the samples used by Kelly & Lang (1970) was insufficiently anaerobic.

The isomer shift and quadrupole splitting of species F1 are very close to those of reduced high-potential Fe iron protein (HiPIP species) of *Chromatium vinosum* ($\delta = 0.42$mm/s; $\Delta E = 1.12$mm/s) (Evans *et al.*, 1970), and the low-temperature Mössbauer spectra suggest that there is no unpaired spin on the iron in each case. Removal of a single electron from the reduced HiPIP species decreased the isomer shift to 0.32mm/s and produced the expected magnetic features in the Mössbauer spectrum, consistent with the unpaired half-integral electron spin being distributed over all four iron atoms (Evans *et al.*, 1970). Species F4, the reduced precursor of F1, is displaced by a similar amount but in the positive direction, having an isomer shift of 0.52mm/s. Its low-temperature spectrum is also consistent with half-integral electron spin delocalized over all four iron atoms. Thus it seems likely that species F1 is produced from F4 by removal of a single electron from the four iron atoms cluster and that species F4 might be regarded as a super-reduced HiPIP species. C. E. Johnson & C. Thompson (personal communication) have independently come to the conclusion that clostridial ferredoxin, containing Fe_4S_4 clusters, has a similar relationship to the HiPIP species. Reduced ferredoxin differs from species F4 in having a much smaller apparent magnetic hyperfine interaction, but reduced Kp2 protein does exhibit the $g = 1.94$ type of e.p.r. signal (Smith *et al.*, 1973) typical of reduced ferredoxins.

The above comparisons suggest that initial O_2 damage to Kp2 protein might be equated with the irreversible oxidation of species F4 to F1. The reason for the irreversibility is not clear, but the Fe_4S_4 clusters of reduced HiPIP species and oxidized ferredoxin are apparently indistinguishable by

X-ray crystallography (Carter *et al.*, 1972), although reduced HiPIP species cannot be reduced further. Thus O_2 inactivation might not be accompanied by destruction of the iron–sulphur cluster structure but by a more subtle change rendering the protein unable to accept an electron and thus incapable of acting as an electron carrier to Kp1 protein in the enzymic reaction.

ATP with Mg^{2+} had a marked effect on the form of the e.p.r. signal of reduced Kp2 protein (Smith *et al.*, 1973), changing the symmetry from rhombic to axial form. In contrast with this observation ATP with Mg^{2+} had little effect on the Mössbauer spectrum of Kp2 protein at 77°K and 4.2°K, although a small change from $\delta = 0.47$mm/s, $\Delta E = 0.9$mm/s to $\delta = 0.5$mm/s, $\Delta E = 1.0$mm/s was observed in the 195°K spectra.

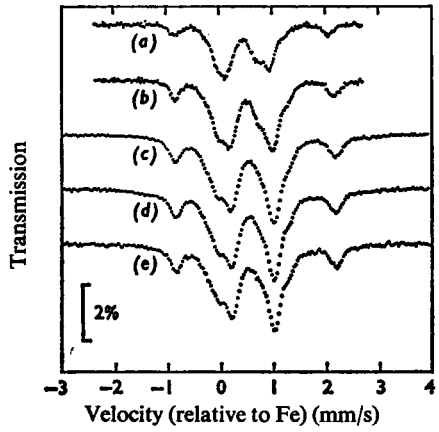

Fig. 7. *Mössbauer spectra of ^{57}Fe-containing Kp1 protein in the presence of ^{56}Fe-containing Kp2 protein, $Na_2S_2O_4$ and the ATP system*

See the Materials and Methods section for details. (*a*) 195°K; (*b*) 77°K; (*c*) 4.2°K, zero magnetic field; (*d*) 4.2°K, 100G magnetic field parallel to the γ-ray beam; (*e*) 4.2°K, 550G magnetic field perpendicular to the γ-ray beam.

Mössbauer spectra of Kp1 and Kp2 proteins together

Kp1 protein. When ^{56}Fe-containing Kp2 protein was added to ^{57}Fe-containing Kp1 protein in the presence of $Na_2S_2O_4$ no change in the Kp1 protein spectrum was observed. However, when the ATP system was added to this protein mixture (the mixture of both proteins, $Na_2S_2O_4$ and the ATP system will be called the N_2-fixation system), rendering it capable of substrate reduction, a marked change occurred (Fig. 7, cf. Fig. 2). We noted earlier that the doublet of species M6 was replaced by a broad shallow absorption, characteristic of magnetic hyperfine interaction, when the temperature was lowered from 77°K to 4.2°K. An inspection of the spectra of Fig. 7 suggested that in the N_2-fixation system this low-temperature magnetic component had largely disappeared and had been replaced by a narrower non-magnetic contribution. In the higher-temperature runs as well, the intensity due to species M6 appeared to be decreased or eliminated, whereas the new component was apparently present. At the same time, species M4 and M5 seemed to be unaffected. To quantify these impressions we attempted a least-squares fit to the spectra of Fig. 7. In each case the quadrupole splitting and isomer shifts for species M4, M5 and M6 were fixed at values taken from the fits to Fig. 2, for the Kp1 protein in its $Na_2S_2O_4$-reduced form. For the 4.2°K spectra the quadrupole splittings were taken to be the same as at 77°K, and 0.02mm/s was added to the isomer shifts to account for the second-order Doppler shift. A fourth quadrupole pair was allowed, with varying quadrupole splitting and isomer shift, and all intensities and line widths were allowed to vary. The results are shown in Table 2. These are much less consistent than those in Table 1, as might be expected when so many unresolved lines are present. However, the new component, which we call species M7, shows a reasonable

Table 2. *Least-squares resolution of the spectra of Kp1 protein in the N_2-fixation system*

Isomer shift, δ, and quadrupole splittings, ΔE, of components M4, M5 and M6 have been fixed at values from Table 1. The three analyses at 195°K refer to the same data and very slightly different starting values. They indicate that the intensities are poorly determined.

		Species M7					
Temperature	Magnetic field	δ (mm/s)	ΔE (mm/s)	Atoms	No. of M6 atoms	No. of M5 atoms	No. of M4 atoms
195°K	0	0.39	0.83	5.7	3.2	6.9	2.2
		0.39	0.81	5.7	3.1	7.0	2.1
		0.38	0.85	3.4	4.1	8.2	2.2
77°K	0	0.42	1.04	3.9	2.8	9.2	2.2
4.2°K	0	0.46	1.05	7.9	1.1	6.4	2.5
	∥	0.45	1.04	7.7	0.5	7.3	2.5
	⊥	0.46	1.04	8.7	0.3	6.5	2.5

trend with temperature as far as δ and ΔE are concerned. Further, it appears to have gained its intensity at the expense of species M6. The intensity of species M6 is extremely low at 4.2°K, as expected, since this component becomes magnetic and indistinct. The relative increase at 4.2°K of species M4, the one well-resolved component, reflects the fact that recognizable intensity had been lost via the magnetic transformation of species M6.

It is useful at this point to compare the Mössbauer data with the e.p.r. data of Smith *et al.* (1972, 1973). $Na_2S_2O_4$-reduced Kp1 protein exhibited an e.p.r. signal with g values near 4.3, 3.7 and 2.01. This signal disappeared on oxidation of the protein with Lauth's Violet and its intensity was also diminished in the N_2-fixation system. On the basis of rapid-freezing and manual e.p.r. experiments Smith *et al.* (1972, 1973) concluded that the decrease in signal intensity in the N_2-fixation system corresponded to a further reduction of Kp1 protein rather than an oxidation.

The e.p.r. signal was associated with the iron atoms of Kp1 protein but was difficult to integrate quantitatively (Smith *et al.*, 1973). Palmer *et al.* (1972), investigating the corresponding e.p.r. signal from the Mo–Fe protein of *Clostridium pasteurianum* (Cp1), found that it integrated to about 0.5 electron/molecule of protein (although they also were doubtful of the validity of the integration) and assigned it to a $S = \frac{3}{2}$ system subjected to both axial and rhombic zero-field splittings. They also associated the e.p.r. signal with the iron atoms of the protein and concluded that these must be in a polynuclear exchange-coupled cluster of some sort, since the $S = \frac{3}{2}$ state is rarely found for mononuclear iron.

The Mössbauer data indicate that species M6 corresponds to eight iron atoms in Kp1 protein. Since this protein consists of two pairs of two types of subunit with molecular weights of about 50000 and 60000 (Eady *et al.*, 1972), species M6 probably represents two Fe_4S_4 clusters. It is a magnetic species and is therefore likely to be e.p.r.-active, and it is transformed into species M7 in the N_2-fixation system, i.e. under the same conditions that led to a decrease in the intensity of the e.p.r. signal from Kp1 protein. The incomplete formation of species M7 from M6 parallels the incomplete formation of e.p.r.-inactive from e.p.r.-active Kp1 protein under similar conditions (Smith *et al.*, 1973). Thus there is strong evidence that species M6 gives rise to the e.p.r. signal from $Na_2S_2O_4$-reduced Kp1 protein with g values near 4.3, 3.7 and 2.01.

Against this point of view is the low integration of the e.p.r. signal, which suggests that it corresponds to not more than a single cluster of atoms. However, since this integration on protein from another source (Palmer *et al.*, 1972) is of doubtful validity and no other change in the iron of Kp1 protein was observed in the N_2-fixation system, we favour the view that species M6 and the e.p.r.-active species are identical.

We have been struck by the similarity between the species M6–M7 transformation and that between the oxidized and reduced species of high-potential Fe protein (HiPIP) of *Chromatium* (Evans *et al.*, 1970). At 77°K the spectrum of the oxidized HiPIP species ($\delta = 0.32$ mm/s; $\Delta E = 0.79$ mm/s) is reasonably close to that of species M6 ($\delta = 0.37$ mm/s; $\Delta E = 0.70$ mm/s), whereas that of the reduced HiPIP species ($\delta = 0.42$ mm/s; $\Delta E = 1.12$ mm/s) is close to that of species M7 ($\delta = 0.42$ mm/s; $\Delta E = 1.04$ mm/s). Further, many features of the magnetic parts of the spectra of Fig. 2 are similar to those found in the oxidized HiPIP species spectra (Evans *et al.*, 1970). We draw particular attention to the strong absorption at -1.2 mm/s and the relative lack of absorption at 0.7 mm/s in the spectrum taken with parallel applied field. Also, the strong absorption at $+1.0$ mm/s in perpendicular field shows up in our Fig. 2 as the deepening of the absorption line of species M5 in that region, relative to the parallel field spectrum. Thus we consider that there is a reasonable case, on the Mössbauer evidence, for the view that the species M6 spectrum represents two Fe_4S_4 clusters resembling oxidized HiPIP.

Unfortunately the nature of the iron atoms of HiPIP species remains unresolved. It is known that oxidation corresponds to the removal of a single electron from the cluster and that in both oxidized and reduced states the four iron atoms have the same quadrupole splitting and isomer shift. In the oxidized state, as in species M6, unpaired half-integral spin is present on all iron atoms. Evans *et al.* (1970) interpreted the magnetic spectrum of the oxidized HiPIP species in terms of the sum of two types. The most recent published X-ray data (Carter *et al.*, 1972) show no distinction between the iron atoms; however, the proton-magnetic-resonance experiments of Phillips *et al.* (1970) indicate the presence of non-equivalent types of iron atom. The distortion of the iron–sulphur cluster required to produce such non-equivalence may be too subtle to be detected at the present resolution of the X-ray data.

The Mössbauer parameters of species M6 and oxidized HiPIP species are not identical, and a further point of difference is in the e.p.r. spectra. We have associated species M6 with the e.p.r. signals with g values near 4.3, 3.7 and 2.01 observed from Kp1 protein (Smith *et al.*, 1972, 1973), whereas oxidized HiPIP species exhibited an axially symmetric e.p.r. signal with $g = 2.12$, $g_\perp = 2.04$ (Palmer *et al.*, 1967).

Kp2 protein. The e.p.r. experiments of Smith *et al.* (1973) showed that Kp2 protein became partially oxidized in the N_2-fixation system. The Mössbauer

Table 3. *Mössbauer spectral species observed in undamaged Kp1 and Kp2 proteins*

Species	Number of Fe atoms/molecule	Temperature (°K)	δ (mm/s)	ΔE (mm/s)	Assignment and comparisons	Conditions for observation
Kp1 protein						
M1	Variable but at least 12, possibly all	77	0.37	0.75	High-spin ferric–high-spin ferrous coupled pairs or higher clusters	Lauth's Violet-oxidized
		4.2	Magnetic			
M2	Variable, possibly all	77	0.37	0.75	High-spin ferric coupled clusters	Lauth's Violet-oxidized
		4.2	0.35	0.9		
M4	2	195	0.60	2.92	High-spin ferrous, spin-coupled, pair of atoms	$Na_2S_2O_4$-reduced and N_2-fixation system
		77	0.66	3.02		
		4.2	0.65	3.05		
M5	8	195	0.56	0.83	Low-spin ferrous clusters	$Na_2S_2O_4$-reduced and N_2-fixation system
		77	0.61	0.83		
		4.2	0.63	0.83		
M6	8	195	0.32	0.69	Similar to oxidized HiPIP species	$Na_2S_2O_4$-reduced
		77	0.37	0.71		
		4.2	Magnetic			
M7	Derived from species M6, up to 8	195	0.39	0.83	Similar to reduced HiPIP species	N_2-fixation system
		77	0.42	1.04		
		4.2	0.46	1.04		
Kp2 protein						
F4	4	195	0.50	0.9	Similar to reduced clostridial ferredoxin	$Na_2S_2O_4$-reduced
		77	0.52	1.05		
		4.2	Magnetic			

spectra of approximately equimolar mixtures of Kp2 protein (containing ^{57}Fe) and Kp1 protein (containing ^{56}Fe) appear identical with those of Kp2 protein alone at 195°K and 77°K, but at 4.2°K the broad magnetic spectrum was overlaid with a pair of small peaks at 0 and 1.0 mm/s. Addition of the ATP system seemed to increase the contribution of these two peaks, and they might be interpreted as being due to an oxidized form of Kp2 protein. However, the natural abundance of ^{57}Fe (2.24%) in the Kp1 protein used in these experiments would in fact contribute approx. 10% of the total spectrum, mainly in the general area of these two peaks. Thus we prefer to reserve any quantitative interpretations of the data until the experiments have been repeated with Kp1 protein from cells grown on pure ^{56}Fe.

Effect of reducible substrates. Kelly & Lang (1970) found that in N_2-fixing mixtures the intensity of the species F2 of Kp2 protein varied with substrate. We have shown above that species F2 is due to a reduced, damaged form of Kp2 protein (and possibly also Kp1 protein), so their observation probably has little relevance to substrate binding to nitrogenase. In low-temperature equilibrium-dialysis experiments they found that the binding of the reducible substrate KCN to mixtures of the two proteins was enhanced when the ATP system was added. We therefore measured the spectra of solutions containing Kp1 (containing ^{57}Fe)+Kp2 (containing ^{57}Fe), Kp1 (containing ^{57}Fe)+Kp2 (containing ^{56}Fe) or Kp1 (containing ^{56}Fe)+Kp2 (containing ^{57}Fe) proteins, in the presence of $Na_2S_2O_4$ and the ATP system, prepared in the presence of Ar, N_2, C_2H_2 or the inhibitor CO. We observed no substrate or inhibitor effects on the iron atoms of either protein.

CO apparently did not prevent the conversion of species M6 into M7. Smith *et al.* (1973), in e.p.r. studies, found that C_2H_2 displaced a pK_a of Kp1 protein. Our results do not contradict this observation since we have not studied the effect of pH on the Mössbauer spectrum of Kp1 protein.

Conclusion

Structural assignments of the spectral species

For ease of reference the isomer shifts and quadrupole splittings of the various species associated with undamaged Kp1 and Kp2 proteins are listed in Table 3, together with our assignments and comparisons with other systems.

The spectra of Kp1 protein reported here are almost completely equivalent to those reported by Kelly & Lang (1970), once the question of oxidation state has been resolved. All of the iron atoms in this protein seem to be grouped in pairs or higher multiples. We have assigned three different iron groupings and three different oxidation states of undamaged Kp1 protein (Table 4). None of these structures seems to be equivalent to those found in ferredoxins, which also contain equivalent numbers of iron and acid-labile sulphur atoms.

Table 4. *Iron species present in the three undamaged oxidation states of Kp1 protein*

Lauth's Violet-oxidized	$Na_2S_2O_4$-reduced (e.p.r.-active)	N_2-fixation system
	M4	M4
M1	M5	M5
M2	M6	M7

($Na_2S_2O_4$ from M1/M2 to M5/M6; Kp2 protein, ATP, Mg^{2+}, $Na_2S_2O_4$ from M5/M6 to M4/M5/M7)

Species F4, which was not observed by Kelly & Lang (1970), is the only species that we can unequivocally assign to undamaged Kp2 protein. Since the function of Kp2 protein is to transfer electrons to Kp1 protein, an active oxidized form presumably exists, but we cannot with certainty describe its Mössbauer spectrum.

Mechanistic conclusions

The observation that formation of species M7 from M6 occurs only in the presence of the N_2-fixation system is of considerable interest, since it is an exclusive feature of this system and shows clearly that iron is intimately involved in the enzymic reaction. However, since different reducible substrates and the inhibitor CO failed to influence the parameters or intensity of species M7, we conclude that it is unlikely to be directly involved in substrate binding. Nor is it likely that species M7 constitutes the hydrogen-evolution site. Carbon monoxide does not inhibit hydrogen evolution and therefore would not be expected to affect this site, but other substrates inhibit hydrogen evolution, and even if this is only by diverting electrons from that site, one might expect the presence or absence of reducible substrates to affect the extent of the species M6 → species M7 transformation.

It therefore seems probable that formation of species M7 from M6 is an important stage in the transfer of electrons to the N_2-binding site. We cannot rule out the possibility that iron is involved in this site or the hydrogen-evolving site, since it may be that in the steady state only small concentrations of substrate-bound complex exist and thus might easily have passed undetected.

Smith *et al.* (1972) in rapid-freezing e.p.r. experiments on Lauth's Violet-oxidized Kp1 protein found that reduction of Kp1 protein was slow except in the N_2-fixation system. They concluded that Kp2 protein acts as an ATP-activated electron carrier to Kp1 protein.

Similar conclusions can be drawn from the Mössbauer experiments. Reconsideration of the data of Kelly & Lang (1970) in the light of our new understanding of the oxidation states of Kp1 protein reveals that all their Kp1 protein spectra were of the oxidized protein except in two cases: (1) after incubation for 1h with 20mg of $Na_2S_2O_4$/ml; (2) in the presence of the N_2-fixation system. Thus reduction of oxidized Kp1 protein occurred either slowly, with $Na_2S_2O_4$ alone, or rapidly, in the time between mixing and freezing the sample, in the N_2-fixation system. No combination of the components of the N_2-fixation system, other than the complete system, resulted in rapid reduction of Kp1 protein. We have confirmed that the reduction of Lauth's Violet-oxidized Kp1 protein by $Na_2S_2O_4$ is slow and have shown that further changes occur, within 1min of mixing, in the N_2-fixation system. We have not repeated the reduction, in the N_2-fixation system, of Lauth's Violet-oxidized Kp1 protein, since we do not believe that this form of the protein is of physiological importance. We conclude, in confirmation of the e.p.r. experiments (Smith *et al.*, 1972, 1973), that Kp2 protein acts as an electron carrier from $Na_2S_2O_4$ to Kp1 protein and that ATP and Mg^{2+} are essential for rapid reaction.

If we assume that, within this class of species, we can correlate Mössbauer spectra with redox potential, then the above role for Kp2 protein is consistent with our structural assignments. We have concluded that species F4 of Kp2 protein is similar to reduced clostridial ferredoxin or a super-reduced HiPIP species. The species M6 → species M7 transformation in Kp1 protein with the N_2-oxidation system is spectrally very similar to the oxidized → reduced transformation of the HiPIP species. Thus Kp2 protein on our assignments would have a lower potential than Kp1 protein and should be able to reduce species M6 to M7.

In the introduction we described the conflicting mechanistic theories of Smith *et al.* (1972, 1973) and of Mortenson *et al.* (1973), the basic difference being that the former group considered that in the N_2-fixation system the Mo–Fe protein cycled between the $Na_2S_2O_4$-reduced form and a 'super-reduced' form, whereas the latter group postulated that it cycled between the $Na_2S_2O_4$-reduced form and a more oxidized form. In corresponding Mössbauer experiments we observed the species M6 → species M7 transformation, and in order to decide which of the above two mechanisms is correct we must decide whether the formation of species M7 from M6 corresponds to an oxidation or a reduction.

Clearly, species M7 is not present in the Lauth's Violet-oxidized form of Kp1 protein. In addition, in our experiments on the progressive oxidation of Kp1 protein we observed no changes in the spectra that could be attributed to the formation of species M7 from M6. However, there are two pieces of evidence in favour of the species M6 → species M7 change being a reduction. First, the observed increase in isomer shift from $\delta = 0.37$mm/s at 77°K for species M6 to $\delta = 0.42$mm/s for species M7 is that expected for an increase in $3d$ electron density, i.e. a reduction.

Secondly, we have a fairly reasonable protein model for the reduction; this is the well-characterized reduction of HiPIP species.

We therefore conclude, in agreement with Smith *et al.* (1972, 1973), that $Na_2S_2O_4$-reduced Kp1 protein becomes further reduced in the N_2-fixation system before passing electrons on to N_2.

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