Evidence on intramolecular electron transfer in the MoFe protein of nitrogenase from *Klebsiella pneumoniae* from rapid-freeze electron-paramagnetic-resonance studies of its oxidation by ferricyanide

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(Received 8 July 1982/Accepted 21 September 1982)

A transient e.p.r. signal with g -values of 2.05, 1.95 and 1.81 was observed in rapid-freezing experiments when Kp1, the MoFe protein of nitrogenase from *Klebsiella pneumoniae*, was oxidized by ferricyanide or by some dyes. This e.p.r. signal was assigned to the 'P'-centres and, since such signals are characteristic of $[4\text{Fe}-4\text{S}]^{1+}$ clusters, provides further evidence for the 'P'-centres being in the $[4\text{Fe}-4\text{S}]^0$ oxidation level in the dithionite-reduced protein. When 4–10-fold excesses of ferricyanide were used as oxidants, the rate of disappearance of the transient e.p.r. signal was independent of the concentrations of ferricyanide, Kp1 or ferrocyanide, i.e. its disappearance was by an intramolecular process. Under some circumstances the $g = 3.7$ e.p.r. signal from the FeMo-cofactors disappeared at a similar rate. It was concluded that, in these circumstances, the $g = 3.7$ e.p.r. signal disappears, owing to intramolecular electron transfer to the 'P'-centres in the $[4\text{Fe}-4\text{S}]^{2+}$ (P^{2+}) oxidation level, whereas the $g_{\text{av.}} = 1.933$ transient e.p.r. signal from the P^{1+} centres disappears, owing to a change in its spin state from $S = \frac{1}{2}$ to $S = \frac{3}{2}$ the rate of this process being maximal when there are two P^{1+} centres in the half-molecule. The rate of the intramolecular decay of the e.p.r. signals, $4.1 \pm 0.8 \text{ s}^{-1}$, is the same as the rate of enzyme turnover. It is suggested that both processes may be linked to the same conformational change, which triggers, or is triggered by, intramolecular electron transfer.

Nitrogenase consists of two metalloproteins, the Fe protein and the MoFe protein, both of which are essential for activity (Eady & Smith, 1979; Mortenson & Thorneley, 1979). Mössbauer (Smith & Lang, 1974) and e.p.r. (Smith *et al.*, 1973) studies have shown that the larger MoFe protein (Kp1 from *Klebsiella pneumoniae*) contains the enzyme's site(s) for binding reducible substrates.

Kp1 is an $\alpha_2\beta_2$ tetramer with a native molecular weight of 218 000, and consists of subunits of molecular weights approx. 50 000 and 60 000. The most active preparations have been reported to contain 2.01 Mo and 32 ± 3 Fe atoms per mol (Smith *et al.*, 1976). Mössbauer spectroscopy

identified four Fe species in the dithionite-reduced Kp1 termed M4, M5, M6 and S (Smith & Lang, 1974; Münck *et al.*, 1975). Species M4 and M5, combined in a ratio of 1:3, are thought to constitute unusual 4Fe–4S clusters, the 'P' clusters (Zimmermann *et al.*, 1978; Kurtz *et al.*, 1979). In the dithionite-reduced protein, all the Fe atoms of the 'P' clusters have isomer shifts typical of ferrous atoms in a tetrahedral environment. This evidence, taken with their diamagnetism, suggests that 'P' clusters in dithionite-reduced MoFe proteins can be assigned as $[4\text{Fe}-4\text{S}]^0$, although this oxidation level is very difficult to reach in model compounds (Smith & Lang, 1974; Zimmermann *et al.*, 1978).

The M6 Fe atoms are combined with the Mo atoms in the FeMo-cofactors (FeMoco) which are responsible for the characteristic S (spin state) = $\frac{3}{2}$ e.p.r. signal, with g -values near 4.3, 3.7 and 2.01, observed with dithionite-reduced proteins (Smith & Lang, 1974; Rawlings *et al.*, 1978). These FeMoco clusters can be extracted into *N*-methylformamide from the purified MoFe protein and will activate

Abbreviation used: FeMoco, FeMo-cofactors.

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various mutants of nitrogen-fixing bacteria that are unable to synthesize FeMoco (Shah & Brill, 1977). Analyses of the extracted FeMoco preparations give Mo/Fe ratios of 1:7–8 (Shah & Brill, 1977; Smith, 1980; Burgess *et al.*, 1980), whereas e.p.r. and Mössbauer studies (Rawlings *et al.*, 1978) have indicated that only six Fe atoms per Mo atom are associated with the unpaired spin. The extraction of an *inactive* MoFe cluster with Mo/Fe of 1:6, which nevertheless in the presence of *N*-methylformamide retained the $S = \frac{3}{2}$ e.p.r. signal (Shah & Brill, 1981), indicated that the extra Fe in *active* FeMoco may be the 'S' Fe species.

Thus the best formulation available for the metal clusters in the MoFe protein is that each half molecule (one of each type of subunit) contains one FeMoco with one Mo atom and seven to eight Fe atoms, and two 'P'-clusters each with four Fe atoms. This stoichiometry has been questioned (Smith *et al.*, 1980; Watt *et al.*, 1981), but it remains the best available description of MoFe-protein structure (Smith, 1982).

Redox processes of isolated Kp1 have been monitored by e.p.r. (O'Donnell & Smith, 1978) and Mössbauer (Smith *et al.*, 1980) spectroscopy. The 'P'-clusters are oxidized at -340 mV (pH 8.7), apparently by one electron per cluster, to a paramagnetic, e.p.r.-silent, oxidation level. Low-temperature magnetic-circular-dichroism studies indicated that, at this oxidation level, the clusters have a ground-state spin of $\frac{5}{2}$ (or $\frac{3}{2}$) (Johnson *et al.*, 1981). The FeMoco are oxidized by one electron per Mo atom to a diamagnetic oxidation level at -216 mV (pH 8.7) in a pH-dependent process (O'Donnell & Smith, 1978). Further reduction of the MoFe proteins has only been achieved during turnover by the MgATP-activated Fe protein when the FeMoco are reduced to an e.p.r.-silent state (Smith *et al.*, 1973) with integer ground-state spin (Huynh *et al.*, 1980).

Little is known about electron transfer into, out of, and within, the MoFe protein, although such knowledge is clearly fundamental to our understanding of the enzymic reduction of N_2 . In the present paper we describe experiments on the kinetics of oxidation of Kp1 by $Fe(CN)_6^{3-}$, which bear on this topic.

Materials and methods

Chemicals

Chemicals and biochemicals were obtained from BDH and Sigma (both of Poole, Dorset, U.K.) respectively. AnalaR potassium ferricyanide was used as oxidant.

Protein

Klebsiella pneumoniae M5a1 was grown and the

nitrogenase proteins purified and assayed as described previously (Eady *et al.*, 1972; Smith *et al.*, 1976). Kp1 was concentrated by binding it to a minimal quantity of DEAE-cellulose, followed by elution with 90 mM-MgCl₂ in 50 mM-Tris/HCl, pH 7.4, containing 2 mM-Na₂S₂O₄ and 0.1 mg of dithiothreitol/ml. Before rapid-freeze experiments, Na₂S₂O₄ and dithiothreitol were removed and the Kp1 equilibrated with 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.4, containing 10 mM-NaCl by passage down a Sephadex G-25 column from which O₂ was rigorously excluded.

After this treatment Kp1 had a specific activity of 1100–1550 nmol of acetylene reduced/min per mg. The activity of the protein was checked before each experiment and also afterwards on the residual protein in the rapid-freeze syringe. No loss of activity was observed during the experiments reported here.

Kp1 with the FeMoco selectively enriched with ⁵⁷Fe was prepared by exploiting a *nifB* mutant of *K. pneumoniae*. This mutant produced Kp1 deficient in FeMoco (Hawkes & Smith, 1983), from which active Kp1 was prepared by reaction with FeMoco prepared from cells grown with ⁵⁷Fe. In oxidation experiments this hybrid protein was compared with another hybrid protein prepared in the same way with unlabelled FeMoco.

Rapid-freeze e.p.r. experiments

These experiments were carried out with full anaerobic precautions as described by Bray *et al.* (1973). Reduced Na₂S₂O₄-free Kp1 from one syringe was allowed to react with K₃Fe(CN)₆ in anaerobic buffer from the other syringe and the reaction solution frozen rapidly in isopentane at -140°C after various reaction times. The resultant 'snows' were packed into 3 mm-diameter e.p.r. tubes and e.p.r. spectra measured with a Varian E9 spectrometer fitted with a variable-temperature probe (Lowe *et al.*, 1972). E.p.r. running conditions were as described in the Figure legends.

Analysis of data

Reaction curves were obtained by plotting the measured heights of a spectral feature (corrected for tube diameter) against the time elapsed between mixing the reactants and freezing them in isopentane. These curves were analysed by least-squares fitting to a minimal number of simultaneous exponentials by using the NAG subroutine D02ABF on a PDP11/34 computer.

Results and discussion

When dithionite-free Kp1 was allowed to react with excess oxidant in rapid-freeze experiments, a

transient e.p.r. signal with g -values of 2.05, 1.95 and 1.81 (Fig. 1a) was observed. The same spectrum was observed when $\text{Fe}(\text{CN})_6^{3-}$ or the dyes phenazine methosulphate and Methylene Blue were used as oxidants. This transient e.p.r. signal with a $g_{\text{av.}}$ of 1.933 is typical of those observed with reduced ferredoxins or from $[\text{4Fe-4S}]^{1+}$ model cluster compounds (Holm, 1977).

Fig. 1. E.p.r. spectrum of the transient species observed on oxidation of Kp1 with $\text{Fe}(\text{CN})_6^{3-}$

(a) Kp1 ($32.8 \text{ mg} \cdot \text{ml}^{-1}$) was allowed to react with a 5-fold molar excess of $\text{K}_3\text{Fe}(\text{CN})_6$, and the solution frozen after 11 ms reaction time. The e.p.r. spectrum was recorded at 19.6 K with a microwave frequency of 9.21 GHz and microwave power of 20 mW using 1 mT field modulation at 100 KHz. (b) ^{57}Fe -enriched Kp1 ($31 \text{ mg} \cdot \text{ml}^{-1}$) was allowed to react with a 10-fold molar excess of $\text{K}_3\text{Fe}(\text{CN})_6$ and the solution frozen after 51 ms reaction time. The e.p.r. spectrum was recorded as in (a), but at 17.5 K and with 0.5 mT field modulation. (c) As (b) but with Kp1 ($17 \text{ mg} \cdot \text{ml}^{-1}$) prepared from *NifB*-Kp1 and $^{57}\text{FeMoco}$. (d) As (b), but with Kp1 ($19.5 \text{ mg} \cdot \text{ml}^{-1}$) prepared from *NifB*-Kp1 and $^{56}\text{FeMoco}$.

Fig. 2 shows the e.p.r. trace obtained from a sample of Kp1 oxidized with ferricyanide and frozen after a reaction time of 12 ms. E.p.r. signals due to FeMoco of the protein (g -values near 4.3 and 3.7), the unchanged $\text{Fe}(\text{CN})_6^{3-}$ (g -value near 2.7), and the transient, are clearly distinguishable. By measuring the time-dependence of the intensities of these signals, it is possible, in principle, to study the kinetics of the reaction. In practice the e.p.r. signal due to each species was measured under different e.p.r. running conditions.

In the present paper we discuss only the changes occurring subsequent to the initial, rapid ($k \sim 100 \text{ s}^{-1}$), appearance of the $g_{\text{av.}} = 1.933$ e.p.r. signal given by Kp1 on oxidation by $\text{Fe}(\text{CN})_6^{3-}$. During the initial, fast, phase the $\text{Fe}(\text{CN})_6^{3-}$ e.p.r. signal intensity decreased rapidly and then remained essentially constant or decreased very slowly during the next 0.5–1 s.

The maximum integration of the $g_{\text{av.}} = 1.933$ signal observed in our experiments was 0.16 electrons/molecule. This was achieved with $\text{Fe}(\text{CN})_6^{3-}$ concentrations from 4- to 10-fold higher than the Kp1 concentration. Lower intensities were observed when only a 2-fold excess of $\text{Fe}(\text{CN})_6^{3-}$ was used. All the $\text{Fe}(\text{CN})_6^{3-}$ e.p.r. signal intensity disappeared within 50 ms in the latter experiments, whereas a residual signal remained when a 4-fold or greater excess of $\text{Fe}(\text{CN})_6^{3-}$ was used.

Fig. 2. E.p.r. signals observed during the reaction of Kp1 with $\text{K}_3\text{Fe}(\text{CN})_6$

Kp1 ($19.6 \text{ mg} \cdot \text{ml}^{-1}$) was allowed to react with an 8-fold molar excess of $\text{K}_3\text{Fe}(\text{CN})_6$ and the solution frozen after 12 ms reaction time. The e.p.r. spectrum was recorded at 11.9 K with a microwave frequency of 9.16 GHz and microwave power of 150 mW using 1.6 mT field modulation at 100 kHz.

Fig. 1(b) shows the e.p.r. signals obtained when ^{57}Fe -enriched Kp1 was used in such oxidation experiments. Comparison with Fig. 1(a) showed that all lines were broadened, indicating that the unpaired electron was associated with an iron-containing species. When Kp1 with FeMoco selectively enriched in ^{57}Fe (see the Materials and methods section) was rapidly oxidized by $\text{Fe}(\text{CN})_6^{3-}$, the signals shown in Fig. 1(c) were obtained. In this case the e.p.r. lines were not broadened (cf. Fig. 1d, the spectrum from comparable protein with $^{56}\text{FeMoco}$). When Kp1 was oxidized to a potential of -270 mV (i.e. the 'P'-clusters oxidized) in the presence of redox-active dyes, the dyes removed, and the protein then allowed to react with a 5-fold excess of $\text{Fe}(\text{CN})_6^{3-}$, no transient e.p.r. signal was observed. These experiments provide strong evidence that the transient e.p.r. signal arises from oxidation of the 'P'-clusters and not FeMoco of Kp1. Furthermore, in some experiments (see Fig. 3), the e.p.r. signal intensity of the dithionite-reduced FeMoco was unchanged whilst the transient e.p.r. signal intensity rose to its maximum. We cannot completely exclude the possibility that the signal is given by the 'S'-centres, but consider it unlikely since these centres are (a) probably part of the FeMoco (Shah & Brill, 1981) and (b) are not oxidized in equilibrium-oxidation experiments. Our assignment of the transient to the 'P'-clusters and their oxidation to give rise to a transient e.p.r. signal characteristic of a $[\text{4Fe-4S}]^{1+}$ cluster is further evidence for the

assignment of the dithionite-reduced form of the 'P'-clusters as $[\text{4Fe-4S}]^0$.

The data of Table 1 show that, when 4–10-fold excesses of ferricyanide were used, the rate of

Fig. 3. Time courses for the decay of the e.p.r. signals from FeMoco and the transient

Kp1 ($22.8\text{ mg}\cdot\text{ml}^{-1}$) was allowed to react with a 5-fold molar excess of $\text{K}_3\text{Fe}(\text{CN})_6$ and the solution frozen after various reaction times. O, Intensity of the $g = 3.7$ e.p.r. signal from FeMoco; □, intensity of the $g_{\text{av}} = 1.933$ e.p.r. signal from the transient. E.p.r. running conditions were as described for Fig. 2 for the $g = 3.7$ e.p.r. signal and as in Fig. 1(b) for the $g = 1.933$ e.p.r. signal. The data are from Expt. 4a in Table 1.

Table 1. Rates of disappearance of the transient $g_{\text{av}} = 1.933$ and FeMoco $g = 3.7$ e.p.r. signals under various conditions

Expt. no.	[Kp1] (mg/ml)	[$\text{Fe}(\text{CN})_6^{3-}$] [Kp1]	Rate of disappearance of e.p.r. signal (s^{-1}) at:		Comments
			$g_{\text{av}} = 1.933$	$g = 3.7$	
1a	18	2	1.1 ± 0.1	Not measured	
2a	19.6	2	0.15	*	
2b	19.6	4	3.4 ± 1.2	5.0 ± 0.1	
3a	18.8	4	3.05	Not measured	
4a	32.8	5	3.9	4.7	
4b	16.4	5	4.05	Not measured	
5a	22.8	5	5.2	$>25 + 5.0$	Contained $\text{K}_4\text{Fe}(\text{CN})_6$ at $4 \times [\text{K}_3\text{Fe}(\text{CN})_6]$
5b	22.8	5	5.2	$>25 + 4.8$	Contained KCl at same ionic strength as $\text{K}_4\text{Fe}(\text{CN})_6$ in 5a
1b	18	5	4.5 ± 0.3	$>25 + 3.9$	
2c	19.6	6	3.4 ± 0.2	4.5 ± 0.5	
2d	19.6	8	3.5 ± 0.2	13	
3b	18.8	10	4.8	Not measured	
6	20	10	5.1	>25	
7	16.7	25	1.5 ± 0.2	$>25^\dagger$	

* The $g = 3.7$ e.p.r. signal decreased by less than 15% in 850 ms reaction time.

† The $g = 3.7$ e.p.r. signal disappeared within 26 ms reaction time.

disappearance of the transient e.p.r. signal, at $4.1 \pm 0.8 \text{ s}^{-1}$ (23°C), was independent of the ferricyanide concentration. Furthermore the rate was also independent of the Kp1 concentration (compare Expts. 4a and 4b, Table 1) and the concentration of the product ferricyanide (compare Expts. 5a and 5b, Table 1). We therefore conclude that, under these conditions, the signal disappeared by an intramolecular process. With a 2-fold or 25-fold excess of $\text{Fe}(\text{CN})_6^{3-}$ over Kp1, the rate of disappearance of the transient signal was slower than the above rate.

In most experiments we also monitored the intensity of the $g = 3.7$ e.p.r. signal associated with the dithionite-reduced FeMoco. With a 2-fold excess of $\text{Fe}(\text{CN})_6^{3-}$ over Kp1, only a 15% change in the intensity of the $g = 3.7$ e.p.r. signal was observed during 850 ms reaction time. When 5–6-fold excesses of $\text{Fe}(\text{CN})_6^{3-}$ were used, the rate of decay of the $g = 3.7$ signal was often, after a short period of no change (Fig. 3), the same as that of the $g_{\text{av.}} = 1.933$ signal; occasionally, however, it was faster or the decay could be analysed as being due to two processes, one with a rate comparable with that of the disappearance of the $g_{\text{av.}} = 1.933$ signal and one faster. This variability could have been due to occasional, inadvertent, partial oxidation of the dithionite-free Kp1 before reaction with the $\text{Fe}(\text{CN})_6^{3-}$, which would lead to behaviour similar to that observed with larger excesses of $\text{Fe}(\text{CN})_6^{3-}$ (Table 1), where the rate of disappearance could either be analysed as a combination of a fast rate with one close to 4 s^{-1} or as a fast rate alone. With a 25-fold excess of $\text{Fe}(\text{CN})_6^{3-}$, all the $g = 3.7$ signal intensity disappeared within 26 ms.

The above data indicate that, in the initial rapid phase of the reaction of $\text{Na}_2\text{S}_2\text{O}_4$ -reduced Kp1 with $\text{Fe}(\text{CN})_6^{3-}$, the first e.p.r.-observable event on the protein is the removal of electrons from the 'P'-clusters. With larger excesses of $\text{Fe}(\text{CN})_6^{3-}$, electrons may also be removed from FeMoco at a rapid rate.

However, under some conditions, the $g = 3.7$ e.p.r. signal intensity from FeMoco decreased at a much slower rate ($k \simeq 4 \text{ s}^{-1}$), equivalent to the rate of decay of the $g = 1.933$ e.p.r. signal of the transient. We have already identified this latter process as intramolecular. Loss of e.p.r. signal intensity occurs by: (1) the centre involved losing or gaining an electron; (2) the centre involved becoming e.p.r.-undetectable, but still paramagnetic as a result of a spin-state change; or (3) two unpaired spins coupling magnetically to give a diamagnetic or e.p.r.-undetectable paramagnetic state.

Clearly, in the observed intramolecular process, FeMoco and 'P'-centres are interacting by one of these three mechanisms. At equilibrium, in titrations with a 6-fold excess of oxidant, the FeMoco each

lose one electron and become diamagnetic and the 'P'-centres also each lose one electron (Zimmermann *et al.*, 1978; Watt *et al.*, 1981) and are oxidized to a $S = \frac{1}{2}$ paramagnetic state (Johnson *et al.*, 1981). The loss of the $g = 3.7$ signal in our experiments is therefore presumably due to the loss of an electron from each FeMoco, i.e. by mechanism (1) above. However, the concomitant disappearance of the transient $g_{\text{av.}} = 1.933$ e.p.r. signal cannot be due to the $[\text{4Fe-4S}]^{1+}$ (P^{1+}) centres accepting this electron, since their equilibrium state is still P^{1+} . In addition, this reaction is thermodynamically unfavourable, since $E_{\text{m}} = -340 \text{ mV}$ for the $\text{P} = \text{P}^{1+}$ reaction and -216 mV for the $\text{FeMoco}^0 = \text{FeMoco}^{1+}$ reaction. The disappearance of the $g_{\text{av.}} = 1.933$ e.p.r. signal must therefore be due to the spin-state change from the $S = \frac{1}{2}$ to the $S = \frac{3}{2}$ state, i.e. mechanism (2) above. We can discard mechanism (3) on the grounds that the equilibrium spin state is not consistent with spin-coupling of two $S = \frac{1}{2}$ centres.

We propose that the acceptor of the electron from FeMoco in the intramolecular process is a P^{2+} -cluster. It is known that 'P'-centres can be further oxidized, without damage, to species giving a complex e.p.r. spectrum with g values between 2.0 and 1.6 (O'Donnell, 1978). This species we tentatively assign as $\text{P}^{1+}\text{P}^{2+}$. The mid-point potential of this oxidation is $+80 \text{ mV}$ and therefore intramolecular electron transfer from FeMoco to P^{2+} centres would be thermodynamically favoured. Furthermore, the intramolecular electron transfer from the FeMoco was only observed when ≥ 4 -fold excess of $\text{Fe}(\text{CN})_6^{3-}$ was allowed to react with Kp1, with the FeMoco e.p.r. signal intensity being initially unchanged (Fig. 3), consistent with the removal of more than one electron from at least one 'P'-centre.

Our data suggest that the rate of the $S = \frac{1}{2}$ to $S = \frac{3}{2}$ spin state change of the P^{1+} -centres was enhanced when both 'P'-centres of a pair were P^{1+} . The rate of the disappearance of the $g_{\text{av.}} = 1.933$ was slower (a) when a 2-fold excess of $\text{Fe}(\text{CN})_6^{3-}$ was used, i.e. in the P^0P^{1+} state, and (b) when a 25-fold excess of $\text{Fe}(\text{CN})_6^{3-}$ was used, i.e. in the $\text{P}^{2+}\text{P}^{1+}$ state. In the latter case the FeMoco in the protein was completely oxidized within 26 ms by direct reaction with oxidant and thus the intramolecular electron transfer could not take place.

To summarize the arguments thus far, we believe that when $\text{Na}_2\text{S}_2\text{O}_4$ -reduced Kp1 is oxidized with $\text{Fe}(\text{CN})_6^{3-}$ (a) Electrons are preferentially removed from the 'P'-clusters until the $\text{P}^{1+}\text{P}^{1+}$ oxidation level is reached; (b) subsequent electrons are removed directly, either from FeMoco or the 'P'-clusters to form FeMoco^{1+} or P^{2+} -clusters respectively; (c) electrons can be transferred intramolecularly from FeMoco^0 to P^{2+} -clusters; (d) the $\text{P}^{1+}\text{P}^{1+}$ oxidation level relaxes from the $S = \frac{1}{2}$ to the $S = \frac{3}{2}$ spin state faster than the P^0P^{1+} or the $\text{P}^{2+}\text{P}^{1+}$ levels.

Scheme 1. Internal electron transfer from FeMoco to the 'P'-clusters and spin relaxation of the 'P'-clusters in Kp1

Steps (c) and (d) are shown in Scheme 1. If the first step, the intramolecular electron transfer, is effectively irreversible, then it must be rate-limiting, otherwise there would be an increase in the concentration of the intermediate which is inconsistent with the data of Fig. 3. Alternatively, if the electron transfer is reversible, then the rate of the spin-state relaxation will be rate-limiting. We favour the former hypothesis, since the measured E_m for the $\text{P}^1 = \text{P}^{2+}$ redox step was +80 mV (O'Donnell, 1978), much higher than that for the $\text{FeMoco}^0 = \text{FeMoco}^{1+}$ redox reaction. However, the measured E_m refers to the equilibrium $S = \frac{1}{2}$ state of the 'P'-clusters and therefore this argument is not conclusive.

The relatively low integration (0.16 electron/molecule) of the transient e.p.r. signal could indicate that it arises from only a small proportion of the Kp1 molecules, which, since the specific activities of our protein preparations were not maximal, might be damaged in some way. However there are several arguments against this hypothesis. Some of these have been detailed above, but for clarity will be summarised as follows.

(a) The e.p.r. signal is typical of $[4\text{Fe}-4\text{S}]^{1+}$ clusters, indicating that it must arise from $[4\text{Fe}-4\text{S}]^0$ clusters. The 'P'-clusters are the only known examples of such clusters in biological systems.

(b) The clusters from which the transient arises have similar redox properties to the 'P'-clusters.

(c) The Mössbauer spectra of the MoFe proteins from three different organisms (Smith & Lang, 1974; Münck *et al.*, 1975; Huynh *et al.*, 1980) are virtually identical, indicating that the ratios of iron-containing species are constant and implying the absence of contaminating iron species. These data are the basis of the all-or-none hypothesis of MoFe protein synthesis [see Smith (1982) for a discussion].

(d) The disappearance of the transient e.p.r. signal is intramolecular and is coupled to the disappearance of the FeMoco e.p.r. signal, indicating that the transient arises from protein containing both

'P'-clusters and FeMoco, i.e. from active Kp1. We therefore conclude that the low integration of the transient e.p.r. signal is because it only arises from active MoFe protein and its appearance is subject to competing decay processes.

Furthermore, in a preliminary communication, Orme-Johnson *et al.* (1981) have reported an e.p.r. signal from Av1 (the MoFe protein from *Azotobacter vinelandii*) similar to the transient reported here, and observed upon careful titration of the reduced Av1 with oxidizing dyes. In this case the signal was maximal after removal of two electrons from the protein and was apparently quite stable. It has also been reported that, in the absence of redox dyes, it was only possible to reduce the 'P'-clusters and not the FeMoco clusters of oxidized Av1 by dithionite (Zimmermann *et al.*, 1978), whereas with oxidized Kp1 both clusters can be reduced by dithionite alone (Smith & Lang, 1974). This would suggest that the observed species difference between Av1 and Kp1 is due to the inability of Av1 to undergo the appropriate conformational change to enable electrons to be transferred between the P- and the FeMoco clusters. This difference may reflect evolutionary pressure causing *Azotobacter* nitrogenase to be more stable to O_2 than *K. pneumoniae* nitrogenase, since *Azotobacter* are obligate aerobes, whereas *K. pneumoniae* only fixes N_2 anaerobically.

The observed intramolecular electron-transfer rate of $4.1 \pm 0.8 \text{ s}^{-1}$ at 23°C is the same, within experimental error, as that of the rate-limiting step in turnover of the enzyme. There is evidence (Thorneley & Lowe, 1981, 1982) that this rate-limiting step in turnover is the dissociation of the oxidized Fe protein from the reduced MoFe protein, whereas we have analysed our data in terms of an intramolecular electron transfer in the absence of the Fe protein. It is possible that the protein-protein dissociation during turnover is coupled to an internal electron transfer in the MoFe protein. Our data on the FeMoco e.p.r. signal show that it need not decay until the transient e.p.r. signal has reached full intensity, i.e. initially it is unaffected by the oxidized 'P'-centres. These data suggest that the electron transfer is probably coupled to a conformational change in the enzyme. Such a conformational change could cause dissociation of the MoFe and Fe proteins during turnover. If the above hypothesis is correct, then clearly Av1 must be able to undergo the appropriate conformational change during turnover, i.e. in the presence of the Fe protein and the effector MgATP.

We thank Mrs. C. Gormal and Mr. M. Reeve-Fowkes for technical assistance, Dr. R. C. Bray for the use of e.p.r. and rapid-freeze equipment provided by the Medical Research Council, Professor J. R. Postgate for comments on the manuscript and Mr. K. Baker, Miss L. Sones and

Mrs. J. Strudwick for growing the *K. pneumoniae* cells. T. R. H. was supported by a Science and Engineering Research Council studentship.

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