A Mössbauer spectroscopic investigation of the redox behaviour of the molybdenum–iron protein from *Klebsiella pneumoniae* nitrogenase

Mechanistic and structural implications

Barry E. SMITH,* Michael J. O'DONNELL,*‡ George LANG† and Kevos SPARTALIAN†§

*A.R.C. Unit of Nitrogen Fixation, University of Sussex, Falmer, Brighton, East Sussex BN1 9RQ, U.K. and

†Department of Physics, College of Science, Pennsylvania State University, 104 Davey Building, University Park, PA 16802, U.S.A.

(Received 24 March 1980/Accepted 13 June, 1980)

The redox properties of the nitrogenase Mo–Fe protein from *Klebsiella pneumoniae* have been monitored by ^{57}Fe Mössbauer spectroscopy between -460 and -160mV (relative to the normal hydrogen electrode). Two redox processes associated with the Fe atoms of the protein were observed. One at -216mV (pH 8.7) was associated with the Fe–Mo cofactor centres in the protein and allowed identification of the Mössbauer parameters of the oxidized form of these centres. The other redox process at -340mV (pH 8.7) was associated with species M5 [Smith & Lang (1974) *Biochem. J.* **137**, 169–180]. This latter redox process may be involved in enzyme turnover. The oxidized form of species M5 interacts magnetically with species M4. The structural implications of the data have been considered in relation to other published data. It is concluded that an unequivocal assignment of the M4 and M5 Fe atoms to Fe–S cluster types is not yet possible.

Nitrogenase (Eady & Smith, 1979; Mortenson & Thorneley, 1979), the enzyme responsible for the biological reduction of N_2 to NH_3 , consists of two metalloproteins, the Fe protein and the Mo–Fe protein. The latter contains the substrate-binding and reducing site of the enzyme. The most active reported preparations of the Mo–Fe protein from *Klebsiella pneumoniae* (Kp1) contained two Mo atoms and 33 ± 3 Fe atoms/molecule (Smith *et al.*, 1976).

Previous Mössbauer spectroscopic studies (Smith & Lang, 1974) on $\text{Na}_2\text{S}_2\text{O}_4$ -reduced Kp1 protein showed the presence of at least three types of Fe atom. Species M4 with isomer shift $\delta = 0.65\text{mm}\cdot\text{s}^{-1}$ and quadrupole splitting $\Delta E = 3.05\text{mm}\cdot\text{s}^{-1}$ at 4.2 K was interpreted as being due to high-spin ferrous

spin-coupled pairs of atoms. Species M5 with $\delta = 0.61\text{mm}\cdot\text{s}^{-1}$ and $\Delta E = 0.83\text{mm}\cdot\text{s}^{-1}$ at 77 K was tentatively assigned as due to low-spin ferrous atoms. Species M6 with $\delta = 0.37\text{mm}\cdot\text{s}^{-1}$ and $\Delta E = 0.71\text{mm}\cdot\text{s}^{-1}$ at 77 K, became a broad, shallow absorption at 4.2 K, indicating magnetic hyperfine interaction and was associated with the e.p.r. signal (g values near 4.3, 3.7 and 2.01) of the Mo–Fe protein. It is now known that species M6 corresponds to the Fe–Mo cofactor centres in the protein (Shah & Brill, 1977; Rawlings *et al.*, 1978).

Later work on $\text{Na}_2\text{S}_2\text{O}_4$ -reduced Av1 protein (Münck *et al.*, 1975) indicated that a fourth type of Fe, designated S, was present in small concentrations. Smith & Lang (1974) showed that oxidation of Kp1 protein by Lauth's Violet changed all the Fe in the protein. O'Donnell & Smith (1978) showed that the e.p.r.-active Fe–Mo cofactor centres of Kp1 protein could be oxidized reversibly in redox titrations to give a pH-dependent $E_m = -220\text{mV}$ at pH 8.7 and -180mV at pH 7.9. In the present paper we describe similar redox titrations monitored by Mössbauer spectroscopy. The aim of this work was to further our understanding of the metal centres in nitrogenase Mo–Fe protein.

Zimmermann *et al.* (1978) reported a Mössbauer spectroscopic investigation of quantitative oxidative

Abbreviations and definitions used: Kp1, Av1 and Cpl are the Mo–Fe proteins of the nitrogenases from *Klebsiella pneumoniae*, *Azotobacter vinelandii* and *Clostridium pasteurianum* respectively. One unit of nitrogenase protein activity is defined as 1nmol of C_2H_2 reduced $\cdot\text{min}^{-1}$ $(\text{mg of protein})^{-1}$.

‡ Current address: Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

§ Current address: Department of Physics, University of Vermont, Burlington, VT 05401, U.S.A.

titrations on Av1 protein. Their data are consistent with ours. Their interpretations will be discussed in more detail in the Results and Discussion sections.

Materials and methods

^{57}Fe -enriched *K. pneumoniae* cells were grown and the nitrogenase proteins were purified by the methods described earlier (Smith & Lang, 1974).

The Mössbauer spectrometer was that described by Lang *et al.* (1976).

Redox titrations were carried out as described by O'Donnell & Smith (1978) and samples were removed from the titration cell and transferred to Mössbauer sample containers as described by Smith & Lang (1974). The reported potentials are relative to the hydrogen electrode.

Sources of gases and chemicals were as given by O'Donnell & Smith (1978).

In the Mössbauer data isomer shifts, δ , and the horizontal axes in the Figures are relative to metallic Fe as standard.

Results

Fig. 1 shows the 195 K Mössbauer spectra of Kp1 protein in the $\text{Na}_2\text{S}_2\text{O}_4$ -reduced and Lauth's Violet-oxidized forms. All of the Fe environments are apparently changed on oxidation. However, the high spin ferrous species M4 in the $\text{Na}_2\text{S}_2\text{O}_4$ -reduced spectrum ($\delta = 0.63 \text{ mm} \cdot \text{s}^{-1}$; $\Delta E = 2.95 \text{ mm} \cdot \text{s}^{-1}$) is changed on oxidation of the protein into two doublets of equal intensity, M4a with $\delta = 0.63 \text{ mm} \cdot \text{s}^{-1}$, $\Delta E = 2.45 \text{ mm} \cdot \text{s}^{-1}$ and M4b with $\delta = 0.6 \text{ mm} \cdot \text{s}^{-1}$, $\Delta E = 3.2 \text{ mm} \cdot \text{s}^{-1}$, which retain the parameters expected of high-spin ferrous Fe. Thus, although the environments of these Fe atoms change, their oxidation states apparently do not.

Fig. 2 shows the 4.2 K Mössbauer spectra of samples of Kp1 protein poised at potentials between -400 mV and -160 mV . The spectrum at -160 mV is the same as that observed by Smith & Lang (1974) for Lauth's Violet-oxidized protein. The spectra at -353 , -288 and -226 mV are clearly intermediate between the two extremes. The lines at $+1.95$ and $+2.2 \text{ mm} \cdot \text{s}^{-1}$ associated with M4a and M4b at 195 K are not apparent in the 4.2 K spectrum of the -160 mV sample, indicating that these ferrous Fe atoms are affected by the magnetic field generated by nearby Fe atoms in the oxidized protein.

The spectrum of the oxidized protein is very complex and as yet we have no detailed understanding of it. In this situation it is extremely difficult to attempt deconvolution. We have instead employed a much simpler analysis, which serves to display the redox dependence of various spectral features. A baseline corresponding to zero absorp-

tion was drawn on each of eight Mössbauer spectra. Then for a particular feature, characterized by a particular Mössbauer velocity, the height of the background-corrected fractional absorption was measured from the baseline. These heights were plotted as a function of potential and the limiting absorption values at the oxidizing and reducing ends were estimated. A difference scale was established by assigning to the lower and higher absorption limits the values 0 and 100% respectively. This is the scale that has been used in presenting the results in Fig. 3. For intense features the 0 and 100% values from individual spectra were within $\pm 5\%$ of the mean, whereas for the weakest features the error increased to approximately $\pm 15\%$.

In Fig. 3(a) two features at $-1.3 \text{ mm} \cdot \text{s}^{-1}$ and $+1.8 \text{ mm} \cdot \text{s}^{-1}$, which have been assigned (Smith & Lang, 1974) to the Fe-Mo cofactor centres, M6, are shown to be relatively constant in intensity to about -280 mV and then to decrease sharply to become

Fig. 1. Comparison of the Mössbauer spectra of $\text{Na}_2\text{S}_2\text{O}_4$ -reduced (a) and Lauth's Violet-oxidized (b) Kp1 protein in 50 mM-Tris/HCl, pH 8.7

Both spectra were measured at 195 K in zero magnetic field.

Fig. 2. Redox titration of Kp1 protein in 50 mM-Tris/HCl at pH 8.7

Samples at various potentials were transferred anaerobically from a redox cell (see the text) to Mössbauer sample containers and were immediately frozen in liquid N_2 . All Mössbauer spectra were measured at 4.2 K with a 0.043 T magnetic field perpendicular to the γ -ray beam. Not all samples are shown. Original sample at (a) -440 mV, (b) -353 mV, (c) -288 mV, (d) -226 mV, (e) -160 mV. Final sample (f) re-reduced to -460 mV. All potentials are relative to the standard hydrogen electrode.

effectively zero at -160 mV. In the same potential range the intensities of two features at -0.05 mm $\cdot$ s $^{-1}$ and $+0.8$ mm $\cdot$ s $^{-1}$ increase. The lines through the points in Fig. 3(a) are theoretical lines for a one-electron redox process. Both the decreasing and increasing features give a mid-point potential of -216 mV at pH 8.7. This corresponds very closely to the mid-point potential of -220 mV at pH 8.7 measured for the loss of intensity of the $g = 3.7$ e.p.r. signal of Kp1 protein (O'Donnell & Smith, 1978), as expected from our assignment of M6. We can thus assign the features at -0.05 and $+1.8$ mm $\cdot$ s $^{-1}$ to the oxidation product of M6. The Mössbauer para-

Fig. 3. Variation with potential of the intensity of the lines from the spectra in Fig. 2

(a) The disappearance of lines associated with species M6 at $+1.8$ (O) and -1.3 (□) mm $\cdot$ s $^{-1}$ compared with the appearance of lines at $+0.80$ (●) and -0.05 (■) mm $\cdot$ s $^{-1}$. (b) The disappearance of lines associated with species M4 at $+2.2$ (O) and -0.85 (□) mm $\cdot$ s $^{-1}$, and species M5 at $+1.0$ (Δ) and $+0.2$ (▽) mm $\cdot$ s $^{-1}$ compared with the appearance of lines at -3.7 (●), -3.0 (■) and $+4.5$ (▲) mm $\cdot$ s $^{-1}$. (c) The appearance of lines at -3.7 (●), -3.0 (■) -2.2 (□), -1.8 (Δ), $+2.8$ (O), $+3.2$ (▽), $+4.5$ (▲) and $+5.0$ (●) mm $\cdot$ s $^{-1}$ with increasing potential.

meters correspond closely to those of the species identified as M2 by Smith & Lang (1974) and Kelly & Lang (1970).

In Fig. 3(b) the changes in the intensities of the lines associated with species M4 at -0.85 and $+2.2$ mm $\cdot$ s $^{-1}$ and with species M5 at $+0.2$ and $+1.0$ mm $\cdot$ s $^{-1}$ are plotted against potential and give a mid-point potential of about -340 mV. The line drawn through these points is a theoretical line for a one-electron process. As the intensities of these lines decrease a number of other lines increase in intensity. The most prominent of these at -3.7 , -3.0 and $+4.5$ mm $\cdot$ s $^{-1}$ are plotted in Fig. 3(b). In Fig. 3(c) the data for these lines are re-plotted together with the data for other features with weaker intensity; it can be seen that all these features increase in intensity with approximately the same

mid-point potential of -340 mV . Thus all the lines associated by Smith & Lang (1974) and Kelly & Lang (1970) with species M1, a complex magnetic species with a large number of lines at 4.2 K, seem to behave similarly in these redox titrations. Furthermore, they can be unequivocally associated with the oxidation products of species M5 together with species M4a and M4b as affected by oxidized M5.

The data in Figs. 3(b) and 3(c) could be fitted equally well with two-electron theoretical curves (not shown).

Discussion

Mechanistic implications

The midpoint potential required to activate nitrogenase is about -460 mV (Evans & Albrecht, 1974; Watt & Bulen, 1976). This potential is presumably that required to reduce the Fe protein which, when activated by MgATP, in turn reduces the Mo-Fe protein. We have argued earlier (O'Donnell & Smith, 1978) that oxidation of the Fe-Mo cofactor centres, which requires a relatively high, species-dependent potential, is unlikely to be important in enzymic turnover. However the potential of -340 mV that we have observed for the oxidation of species M5 is close enough to the potential for nitrogenase function for M5 to become partially oxidized during turnover under conditions of limiting reductant (MgATP-activated Fe protein). Under such conditions, at 10°C , Lowe *et al.* (1978) observed a number of transient e.p.r. signals, at least some of which can therefore be associated with oxidized M5.

Such e.p.r. signals are not observed during redox titrations on isolated Kp1 protein, but a transient e.p.r. signal, corresponding to an Fe-S cluster at the -3 oxidation level, has been observed during the first 1 s of the oxidation of Kp1 protein with $\text{Fe}(\text{CN})_6^{3-}$ (M. J. O'Donnell, B. E. Smith & D. J. Lowe, unpublished work).

Although the above evidence indicates that the electrons on species M5 are available for substrate reduction, it is not yet possible to say whether electrons necessarily pass through species M5 during turnover.

It is believed that the Fe-Mo cofactor centres are the site of substrate binding and reduction, i.e. that they are the point of electron departure from Kp1 protein (Smith, 1977). Electrons from MgATP-activated Kp2 protein reach the Fe-Mo cofactor centres at a rate indistinguishable from the rate of hydrolysis of MgATP (Eady *et al.*, 1978). Thus, either the Fe-Mo cofactor centres are the point of entry of electrons into Kp1 (in which case the M5-containing centres may act as electron buffer centres that are only used under conditions of electron starvation) or alternatively the M5-con-

taining centres may be directly on the electron transfer path from the Fe protein to the Fe-Mo cofactor centres but an earlier step on the path is rate-limiting and so the M5-containing centres never appear to be further reduced.

Structural implications

The data presented here show that the e.p.r.-observable redox process of the nitrogenase Mo-Fe protein (O'Donnell & Smith, 1978) affects only those Fe atoms associated with the Fe-Mo cofactor centres, i.e. species M6, and allow us to identify M2 (cf. Smith & Lang, 1974) as the oxidized form of the Fe in these centres. Although these centres also contain acid-labile sulphur atoms it would probably be unwise to use their parameters for any structural comparison with normal Fe-S clusters, which have markedly different chemical and physical properties (Rawlings *et al.*, 1979).

We have shown that species M5 undergoes a redox process with an E_m near -340 mV at pH 8.7 and that species M4 is affected by the magnetic field associated with oxidized M5. Zimmerman *et al.* (1978) suggested that the M4 and M5 species in the Mo-Fe protein together constitute four 4-Fe 'P' clusters with each cluster containing one M4 and three M5 Fe atoms. One-electron oxidation of each cluster renders the M5 Fe atoms magnetic and thus the M4 species experience the magnetic interaction that is apparent at 4.2 K. This is an attractive idea that deserves serious consideration but it is important to realize the limitations of the currently available data.

The most active preparation of Kp1 protein yet described contained $2.01 \pm 0.1\text{ Mo}$ atoms and $32.5 \pm 3\text{ Fe}$ atoms, in a molecule of mol.wt. 218 000, and had an acetylene-reduction activity of 2150 units (Smith *et al.*, 1976). These metal analyses may still not be maximal since the activity/g-atom of Mo of Fe-Mo cofactor prepared from Kp1 protein extrapolates to a specific activity of 2500–2900 units for a molecule of mol.wt. 218 000 containing 2 Mo atoms (Smith, 1980). Nevertheless recent analytical data on Av1 protein also indicate 2 Mo and 30–36 Fe atoms/molecule (Shah & Brill, 1977) and thus an Fe content in this range is not unreasonable.

The proportions of the different species observed in the Mössbauer spectra of $\text{Na}_2\text{S}_2\text{O}_4$ -reduced Kp1 protein and Av1 protein are shown in Table 1. The original analysis of the Kp1 protein spectra was in terms of the three major species only, i.e. M4, M5 and M6 (Smith & Lang, 1974). Munck *et al.* (1975), with Av1 protein, later demonstrated the presence of the minor species 'S' which contributed about 5–6% to the total absorption. This species can also be identified in the Kp1 protein spectra and so the Kp1 data have been adjusted on the assumptions that species 'S' was responsible for 6% of the total

absorption and contributed equally to the estimates of M5 and M6 absorption and not to the M4 absorption. With these adjustments the spectral analyses for the two proteins are virtually identical (Table 1).

The ⁵⁷Fe-enriched proteins used for Mössbauer spectroscopy, for economic and historical reasons, were not of the highest possible specific activity or Fe content. The Av1 protein had 22 (Zimmerman *et al.*, 1978) and the Kp1 protein about 18 (Smith & Lang, 1974) Fe atoms/molecule. Extrapolation of the Mössbauer species analyses on these proteins to fully active protein with an Fe content of 30–36 Fe atoms/molecule assumes an ‘all-or-none’ mechanism for metal-ion insertion into the Mo-Fe protein; i.e. since the proteins are $\alpha_2\beta_2$ tetramers, that all of the molecules present contain all, exactly half or none of each of their possible metal species and thus the ratio of species present in the experimental preparations was identical with the ratio in fully active protein. This assumption is supported by the similarity of the Kp1, Av1 and Cpl protein (Zimmerman *et al.*, 1978) Mössbauer spectra but can only be regarded as a working hypothesis at this time.

We have considered the possible permutations of the numbers of Fe atoms of each species in the Mo-Fe proteins by using the following assumptions.

(a) That the all-or-none hypothesis is valid (see above).

(b) That the molecules, as $\alpha_2\beta_2$ tetramers, will exhibit 2-fold symmetry and therefore contain even numbers of Fe atoms.

(c) That there are two Fe-Mo cofactor centres, each containing 1 Mo atom and 5–8 Fe atoms, in each molecule (Shah & Brill, 1977; Huynh *et al.*, 1979; Smith, 1980).

(d) That all of the iron species have the same Debye-Waller factors.

(e) That fully active Mo-Fe proteins contain 28–38 Fe atoms/molecule.

These assumptions, together with the percentage compositions from Table 1, limit consideration to molecules that contain 4 M4 and 2 ‘S’ Fe atoms with 10–16 M6 and 12–16 M5 Fe atoms.

Of the possible permutations, four are in reasonable agreement with the experimental data (Table 1). To distinguish between these possibilities further evidence is required.

Zimmerman *et al.* (1978) claim that in oxidative titrations with Lauth’s Violet four electrons per protein molecule can be removed from Av1 protein before the Fe-Mo cofactor centres begin to be oxidized. (Similar experiments with Kp1 protein gave a linear decrease in Fe-Mo cofactor centre e.p.r. signal intensity on oxidation with from one to six oxidizing equivalents). They also considered that species S was unaffected in these oxidative titrations. We have shown that species M4 is not

Table 1. Comparison of observed and calculated contributions of the observed species to the total Mössbauer spectra
See the text for limitations on calculations. Errors are standard deviations.

Species	Observed contributions (%)				Calculated contributions					
	Smith & Lang (1974)*	Munck <i>et al.</i> (1975)	Zimmerman <i>et al.</i> (1978)		Atoms of Fe/molecule	(%)	Atoms of Fe/molecule	(%)	Atoms of Fe/molecule	(%)
M4	12.3 ± 1	14 ± 1	13		4	13.3	4	12.5	4	11.8
M5	41.7 ± 2.9	42.5 ± 3	41		12	40	14	43.75	14	41.2
M6	39.9 ± 3.4	38.5 ± 3	40		12	40	12	37.5	14	41.2
S	6	approx. 5	5–6		2	6.7	2	6.25	2	5.9
				Total atoms of	30		32		34	
				Fe/molecule						
										Atoms of Fe/molecule
										(%)
										4
										16
										14
										2
										36

* Adjusted for 6% contribution from species ‘S’ (see Text).

oxidized under these conditions and therefore the four electrons must come from oxidation of species M5. Therefore the number of M5 Fe atoms/molecule should give an integer when divided by four. On this argument we can reasonably discount the two possible permutations (Table 1) containing 14 M5 Fe atoms/molecule. This leaves two possibilities, containing 30 and 36 Fe atoms/molecule respectively. Before further discussion of the Mössbauer spectroscopy it is useful to consider the results from other techniques on the arrangement of the Fe atoms in the Mo-Fe proteins.

The near i.r. magnetic circular dichroism spectra of the reduced Av1 and Kp1 proteins indicate the absence of 4Fe-4S clusters in the oxidation levels found in other proteins (Stephens *et al.*, 1979).

The technique of cluster extrusion (Holm, 1977) has been applied to a number of Fe-S proteins and enzymes with considerable success in distinguishing and quantifying 2Fe-2S and 4Fe-4S clusters. Unfortunately some difficulty has arisen in application of this technique to the nitrogenase Mo-Fe proteins. Nevertheless Orme-Johnson *et al.* (1977) by using extrusion from Cpl protein into the apoproteins of *Bacillus polymyxa* ferredoxin and adrenodoxin, mediated by *o*-xylyldithiol in aqueous dimethylsulphoxide solution, found evidence for 4Fe-4S clusters only. Similarly Kurtz *et al.* (1979) with the same extrusion agent found evidence for four 4Fe-4S clusters/molecule in Na₂S₂O₄-reduced Cpl and Av1 protein. However, Wong *et al.* (1979), by using *p*-trifluoromethylbenzenethiol as extruding agent in aqueous hexamethylphosphoramide solution, found evidence for 4Fe-4S clusters with the dithionite-reduced protein and also for 2Fe-2S clusters with the Lauth's Violet-oxidized protein. Extrusion of the Fe was incomplete in these experiments.

The apparent absence of 2Fe-2S clusters in the reduced Mo-Fe protein can be rationalized on the basis of the chemistry of these clusters. The non-Fe-Mo cofactor Fe atoms in the reduced Mo-Fe proteins appear to be largely ferrous (Smith & Lang, 1974). It is known (Wong *et al.*, 1979) that reduction of the model 2Fe-2S analogues [Fe₂S₂X₄]²⁻ (X = halide) can lead to dimerization, i.e.

It therefore seems possible that such reactions could be confusing the extrusion results obtained with the reduced Mo-Fe proteins with 2Fe-2S clusters extruding as 4Fe-4S clusters. In summary, in addition to the Fe-Mo cofactor centres there seems to be reasonably good evidence for 4Fe-4S clusters of an unconventional nature in the Mo-Fe proteins and some evidence for 2Fe-2S clusters as well.

Returning to our consideration of the Mössbauer data, Zimmerman *et al.* (1978) favoured the 30-Fe atom alternative of Table 1. In this, each molecule contained four M4 and 12 M5 Fe atoms with these forming four equivalent 4Fe-4S clusters formed from one M4 and three M5 iron atoms. Only species 'S' could form 2Fe-2S clusters.

With the 36-Fe atom alternative of Table 1 the most probable configuration would be with four 4Fe-4S clusters made up entirely from M5 Fe atoms, with the M4 atoms gathered in two 2Fe-2S clusters. One argument against the latter hypothesis is that it is necessary to invoke a magnetic interaction between the one-electron oxidized M5 cluster and the M4 clusters. This would, however, allow a relatively straightforward explanation for the existence of two forms of M4, M4a and M4b, in the oxidized protein if one M4 Fe atom of the 2Fe-2S cluster were closer than the other to the M5 cluster.

Further evidence for the presence of 2Fe-2S clusters consisting of two M4 atoms comes from studies of the magnetic properties of these atoms. They have the Mössbauer parameters expected of high-spin ferrous ions in a tetrahedral sulphur environment, and yet unpaired electron spin has not been detected in Mössbauer experiments on the Na₂S₂O₄-reduced proteins at low temperatures and in high magnetic fields (Smith & Lang, 1974; Munck *et al.* 1975).

An attractive explanation of this phenomenon is that the M4 atoms are grouped in antiferromagnetically spin-coupled pairs (Smith & Lang, 1974). Species M5 has Mössbauer parameters consistent with low-spin ferrous ions, i.e. with no unpaired electrons available for spin-coupling. This assignment (Smith & Lang, 1974) is admittedly tentative but complete antiferromagnetic spin-coupling of the integral spin of the high spin ferrous M4 ions by interaction with three M5 ions would require a very complex spin-coupling scheme. Spin-coupling between M4 atoms in adjacent clusters would explain this phenomenon, but seems inherently less likely than spin coupling within a cluster.

In summary, we have been able to identify the oxidized form of the Fe-Mo cofactor centres and have demonstrated their independence from other redox centres. The nature and importance of the 'S' Fe atoms is still unclear and it seems that on the current evidence we cannot distinguish between the two most likely configurations that we have been considering for the Mössbauer species M4 and M5 in the nitrogenase Mo-Fe proteins. Further experiments will be necessary in order to solve this fascinating problem. It is important to remember that underlying the whole of the above structural arguments is the all-or-none hypothesis. Clearly one important priority is the measurement of the

Mössbauer spectra of proteins with the full complement of Fe.

Part of this work was supported by the National Institutes of Health under grant HL-16860. We thank Professor J. R. Postgate for useful comments on the manuscript.

References

- Eady, R. R. & Smith, B. E. (1979) in *A Treatise on Dinitrogen Fixation* (Hardy, R. W. F., Bottomley, F. & Burns, R. C., eds.), sections I and II, pp. 399–490, Wiley Interscience, New York
- Eady, R. R., Lowe, D. J. & Thorneley, R. N. F. (1978) *FEBS Lett.* **95**, 211–213
- Evans, M. C. W. & Albrecht, S. L. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1187–1192
- Holm, R. H. (1977) in *Biological Aspects of Inorganic Chemistry* (Addison, A. W., Cullen, W. R., Dolphin, D. & Jones, B. R., eds.), pp. 71–111, Wiley, New York
- Huynh, B. H., Munck, E. & Orme-Johnson, W. H. (1979) *Biochim. Biophys. Acta* **527**, 192–203
- Kelly, M. & Lang, G. (1970) *Biochim. Biophys. Acta* **233**, 86–104
- Kurtz, Jr., D. M., McMillan, R. S., Burgess, B. K., Mortenson, L. E. & Holm, R. H. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4986–4989
- Lang, G., Spartalian, K. & Yonetani, T. (1976) *Biochim. Biophys. Acta*, **451**, 250–258
- Lowe, D. J., Eady, R. R. & Thorneley, R. N. F. (1978) *Biochem. J.* **173**, 277–290
- Mortenson, L. E. & Thorneley, R. N. F. (1979) *Annu. Rev. Biochem.* **48**, 387–418
- Munck, E., Rhodes, H., Orme-Johnson, W. H., Davis, L. C., Brill, W. J. & Shah, V. K. (1975) *Biochim. Biophys. Acta* **400**, 32–53
- O'Donnell, M. J. & Smith, B. E. (1978) *Biochem. J.* **173**, 831–839
- Orme-Johnson, W. H., Davis, L. C., Henzl, M. T., Averill, B. A., Orme-Johnson, N. R., Munck, E. & Zimmerman, R. (1977) in *Recent Developments in Nitrogen Fixation* (Newton, W., Postgate, J. R. & Rodriguez-Barrueco, C., eds.), pp. 131–178, Academic Press, London, New York, San Francisco
- Rawlings, J., Shah, V. K., Chisnell, J. R., Brill, W. J., Zimmerman, R., Munck, E. & Orme-Johnson, W. H. (1978) *J. Biol. Chem.* **253**, 1001–1004
- Shah, V. K. & Brill, W. J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3249–3253
- Smith, B. E. (1977) *J. Less-Common Met.* **54**, 465–475
- Smith, B. E. (1980) in *Molybdenum Chemistry of Biological Significance* (Newman, W. & Otsuka, S., eds.), pp. 179–190, Plenum, New York
- Smith, B. E. & Lang, G. (1974) *Biochem. J.* **137**, 169–180
- Smith, B. E., Thorneley, R. N. F., Yates, M. G., Eady, R. R. & Postgate, J. R. (1976) *Proc. Int. Symp. Nitrogen Fixation 1st* **1**, 150–176
- Stephens, P. J., McKenna, C. E., Smith, B. E., Nguyen, H. T., McKenna, M.-C., Thomson, A. J., Devlin, F. & Jones, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2585–2589
- Watt, G. D. & Bulen, W. A. (1976) *Proc. Int. Symp. Nitrogen Fixation 1st* **1**, 248–256
- Wong, G. B., Kurtz, Jr., D. M., Holm, R. H., Mortenson, L. E., & Upchurch, R. G. (1979) *J. Am. Chem. Soc.* **101**, 3078–3090
- Zimmerman, R., Munck, E., Brill, W. H., Shah, V. K., Henzl, M. T., Rawlings, J. & Orme-Johnson, W. H. (1978) *Biochim. Biophys. Acta* **537**, 185–207