Electron-paramagnetic-resonance and magnetic-circular-dichroism studies of the binding of cyanide and thiols to the iron-molybdenum cofactor from *Klebsiella pneumoniae* nitrogenase

Andrew J. M. RICHARDS,* David J. LOWE,† Raymond L. RICHARDS,† Andrew J. THOMSON* and Barry E. SMITH††

*School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.,

and †AFRC Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, U.K.

FeMoco, a low- M_r metal cluster of probable composition Fe_7MoS_9 complexed with homocitrate, has been extracted with *N*-methylformamide from the MoFe protein of the nitrogenase enzyme from *Klebsiella pneumoniae*. The binding of cyanide and thiols to the FeMoco cluster in its paramagnetic $S = 3/2$ oxidation level has been studied by low-temperature e.p.r. and magnetic-circular-dichroism (m.c.d.) spectroscopies. Cyanide binds to isolated FeMoco at more than one site, and causes

changes in the g values from $g = 4.6, 3.2, 2.0$ to $g = 4.29, 3.82, 2.02$. E.p.r. competition studies indicate that one cyanide can be displaced by thiolate from one type of site. The form of the low-temperature m.c.d. spectrum is little changed by ligand binding, thus the basic cluster structure remains intact. However, when benzenethiol is bound, a new intense band (λ 387 nm) is observed, indicating the generation of an increased ligand-to-cluster charge-transfer interaction.

INTRODUCTION

Nitrogenase, the enzyme responsible for biological nitrogen fixation, consists of two oxygen-sensitive metalloproteins: an iron (Fe) protein and a molybdenum-iron (MoFe) protein (Lowe et al., 1985; Eady, 1986; Smith and Eady, 1992), a vanadium-iron (VFe) protein (Robson et al., 1986) or a putative iron-only protein (Chisnell et al., 1988; Pau et al., 1989). The molybdenum-containing system has been studied for a number of years and is relatively well characterized. A structural model based on a crystallographic analysis of the MoFe protein from *Azotobacter vinelandii* has been reported (Kim and Rees, 1992a,b).

The most active preparations of the MoFe protein contain two molybdenum ions and about 30 iron and acid-labile sulphur ions per molecule of M_r about 220 000 [see Smith (1983)]. The molybdenum ions are contained in two iron-molybdenum cofactor (FeMoco) centres which can be extracted from active MoFe protein into *N*-methylformamide (NMF) as a low- M_r metal cluster of probable composition Fe_7MoS_9 (Burgess, 1990; Kim and Rees, 1992a) which also contains homocitrate (Hoover et al., 1989). FeMoco can activate the MoFe protein polypeptides formed by mutants (e.g. *nifB* mutants) that are unable to synthesize FeMoco. *NifV* mutants of *Klebsiella pneumoniae* have an altered substrate specificity (McLean and Dixon, 1981). When the FeMoco from the MoFe protein of these mutants was extracted and used to activate extracts of *nifB* mutants, the altered substrate specificity was also transferred, whereas wild-type FeMoco conferred the wild-type substrate specificity (Hawkes et al., 1984). These experiments provide good evidence that FeMoco is, includes or is part of the substrate-binding and -reducing site of the enzyme. However, direct interactions between the FeMoco centres in the protein and reducible substrates have not been demonstrated.

Smith et al. (1973) showed that the pK_a associated with the

characteristic $S = 3/2$ spin system of protein-bound FeMoco, with e.p.r. signals near $g = 4.3, 3.7$ and 2.01 , was modified by the substrate acetylene. Furthermore, the spin state of the FeMoco centres was changed to $S = 1/2$ during enzyme turnover in the presence of the product ethylene (Lowe et al., 1978; Hawkes et al., 1983), but no evidence of a direct interaction between the FeMoco sites in the protein and acetylene, H^+ or ethylene could be shown. Isolated FeMoco also has an e.p.r. signal characteristic of an $S = 3/2$ spin system (Rawlings et al., 1978; Smith et al., 1985; Newton et al., 1989; Burgess, 1990). We have previously reported that a change to a more nearly-axial form of the $S = 3/2$ e.p.r. signal occurs when cyanide binds to isolated FeMoco (Smith et al., 1985). In the present paper we describe this interaction in detail.

Cyanide is both a substrate and inhibitor of nitrogenase (Hardy and Knight, 1967; Kelly et al., 1967; Li et al., 1982; Lowe et al., 1989). Both HCN and CN^- exist in solution at pH 7, and Li et al. (1982) appeared to have shown, by analysing steady-state data, that HCN was the substrate species and CN^- was the inhibitor. Lowe et al. (1989), however, showed that these results, and also additional pre-steady-state data, could be explained by a single binding site; thus which of CN^- or HCN can bind to nitrogenase and whether there are one or two binding sites is still not fully established. As this point is unclear, we refer throughout this paper to 'cyanide' to indicate either or both species. CO probably binds at the same site(s), as it relieves cyanide-induced inhibition of H_2 formation by nitrogenase. There is considerable interest in determining the site or sites at which cyanide binds. This has proved difficult to investigate in the holoenzyme because of the paucity of spectroscopic techniques with which to observe individual sites at which potential ligands can bind.

In the present paper we provide evidence for a direct interaction between extracted FeMoco and the reducible substrate cyanide by means of e.p.r. and low-temperature magnetic circular

Abbreviations used: NMF, *N*-methylformamide; DMF, dimethylformamide; m.c.d., magnetic circular dichroism; $[\text{NBu}_4]\text{CN}$, tetrabutylammonium cyanide.

† To whom correspondence should be addressed.

dichroism (m.c.d.) spectroscopy (Smith et al., 1985). In a complementary study, the interaction of cyanide and methylisocyanide (CH_3NC) with the FeMoco ligated by the thiolate ligand $p\text{-CF}_3\text{C}_6\text{H}_4\text{SH}$ in NMF was followed by ^{19}F n.m.r. and X-ray absorption experiments (Conradson et al., 1989).

MATERIALS AND METHODS

All chemicals, unless otherwise stated, were purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Growth of organisms, purification of proteins and isolation of FeMoco

Wild-type *K. pneumoniae* M5a1 and the *nifB* mutant Kp5058 were grown and nitrogenase proteins extracted and purified as previously described (Eady et al., 1972; Smith et al., 1976; Hawkes and Smith, 1983).

FeMoco was extracted into NMF from purified *K. pneumoniae* MoFe protein either after precipitation of the protein by dimethyl sulphoxide and washing the precipitate with dimethylformamide (DMF) (Smith, 1980) or by a modification of the method of McLean et al. (1989).

FeMoco activity

FeMoco was assayed by incubating portions of the FeMoco solution with crude extracts of Kp5058 and then assaying the MoFe protein formed for acetylene reduction after adding additional iron protein (Eady et al., 1972; Hawkes and Smith, 1983). One unit of activity is defined as 1 nmol of ethylene produced/min. FeMoco samples used in the current work had activities of 250–290 units/ng-atom of Mo.

Molybdenum analyses were carried out by the method of Clarke and Axley (1955).

Preparation of FeMoco samples for spectroscopy

The NMF and DMF used were bubbled with O_2 -free N_2 and then made 2% (v/v) in water, 5 mM in Na_2HPO_4 , pH 8, and 2 mM in $\text{Na}_2\text{S}_2\text{O}_4$. Concentrated FeMoco solutions were prepared by evaporating an NMF extract to dryness and then redissolving the residue in a known volume of NMF. DMF was then added to make the solutions 2:1 (v/v) NMF/DMF which at low temperature gave good optical glasses for m.c.d. spectroscopy.

E.p.r. samples were frozen to liquid-nitrogen temperatures in quartz e.p.r. tubes anaerobically. The titration of Figure 2 was carried out on a single e.p.r. tube which was returned, frozen, to the glove box for melting and further addition of cyanide before refreezing and remeasuring the spectrum. Repeated titrations using appropriate samples made up in separate e.p.r. tubes gave identical results. Competition experiments were carried out in a similar fashion but in NMF solution.

KCN solutions for titration were made up anaerobically in water/DMF (1:1, v/v) with the measured pH being adjusted to 9.5 with NaOH. The concentration of cyanide was determined by titration with AgNO_3 in ammoniacal solution using KI to detect the end point (Kolthoff and Sandell, 1952). Analytically pure tetrabutylammonium cyanide ($[\text{NBu}_4]\text{CN}$) was used as the source of cyanide in some of the competition experiments as indicated in the text, because of its higher solubility in NMF. In

experiments where both sources of cyanide were used the results were indistinguishable.

E.p.r. spectra were measured, at Norwich (Figures 1 and 2), on a Bruker ER-200C spectrometer fitted with an ESR-9 flow cryostat (Oxford Instruments Limited) and interfaced to an Aspect 2000 computer, or at Sussex (Figures 3 and 4) on a Bruker ER-300SH spectrometer also fitted with an ESR-9 cryostat and interfaced to an ESP 1600 computer. All sample handling was under N_2 in a glove box (Faircrest Engineering, Croydon, Surrey, U.K.) with a gas recirculation system that maintained the O_2 concentration below 1 p.p.m.

Fits to titration and Job plot data were done with an unweighted least-squares procedure using NAG subroutines E04FDF and E04YCF on a microvax 3600 computer.

RESULTS

E.p.r. measurements

The addition of cyanide to NMF/DMF (2:1, v/v) solutions of isolated FeMoco causes a pronounced change in the form of its e.p.r. spectrum (Figure 1). Isolated FeMoco has an e.p.r. spectrum (Figure 1a) with g values near 4.6, 3.3 and 2.0 which is more rhombic than, but clearly related to, the spectrum arising from the FeMoco centres in the MoFe protein. In the presence of cyanide, this spectrum becomes much more axial (Figure 1b) with g values of 4.29, 3.82 and 2.02 and with narrower lines. This spectrum loses intensity rapidly with increasing temperature, as do those of FeMoco and the MoFe protein, being almost

Figure 1 X-band e.p.r. spectra of (a) FeMoco and (b) the cyanide adduct of FeMoco

FeMoco (69 μM) was dissolved in NMF/DMF (2:1, v/v). KCN (3 equiv.) was added to (b). The temperature for e.p.r. measurements was 5 K and the microwave power was 2 mW at 9.42 GHz. Field modulation was 1.9 mT at 100 KHz.

Figure 2 X-band e.p.r. spectra of FeMoco plus KCN as a function of cyanide concentration

FeMoco (69 μ M) was dissolved in NMF/DMF (2:1, v/v). Equivalents of KCN in water/DMF (1:1, v/v) added were 0, 0.228, 0.586, 0.857, 1.27, 1.63, 2.46 and 3.20. E.p.r. conditions were as in Figure 1.

undetectable above 20 K. The presence of cyanide caused no loss in FeMoco activity implying that binding was fully reversible under assay conditions.

The spectrum of FeMoco in the presence of cyanide is similar in form to published simulations of an $S = 3/2$ system with

$D > 0$ and a small rhombicity (Collison and Mabbs, 1982). The retention of activity and the nature of the e.p.r. change imply that cyanide acts as a ligand which causes only an alteration in the zero-field splitting of the ground state of FeMoco rather than any major structural change.

To quantify this result, changes in the e.p.r. spectrum were followed during a titration with cyanide. The resulting spectra are plotted in Figure 2. Isoclinic points and only two major spectral forms, those of free FeMoco and the cyanide-bound species, were observed. An estimation of the percentage of cyanide-bound species was obtained by measuring the variation of the peak-to-peak height of the $g = 3.82$ feature which only has contributions from the cyanide-bound species. A plot of the percentage of bound species against equivalents of cyanide added is presented in Figure 3(a). We have also studied this interaction by means of a Job titration, which is shown in Figure 3(b). Both techniques give a non-integer binding stoichiometry close to 1.5 $[\text{NBu}_4]\text{CN}$ per Mo in FeMoco.

It is well established that isolated FeMoco binds one molecule of thiol per Mo (Rawlings et al., 1978) resulting in a decrease in rhombicity and linewidth of the e.p.r. spectrum, thus making it more like that observed from the protein, in which FeMoco is bound at Fe by one cysteine residue (Kim and Rees, 1992a). We therefore studied the relative binding abilities of cyanide and PhS^- by competition experiments, monitored by e.p.r., as shown in Figure 4. A solution of dithionite-reduced FeMoco in NMF was treated with $[\text{NBu}_4]\text{CN}$ (2.7 mol/Mo) giving the characteristic spectrum (Figure 4c). Addition of PhSH (2 mol/Mo) caused the spectrum (Figure 4e) to change to that typical of PhS^- -bound cofactor (Figure 4b) (Rawlings et al., 1978). On the other hand, if a solution of FeMoco containing a two molar excess of PhSH per Mo was treated with 2.7 mol of $[\text{NBu}_4]\text{CN}$ per Mo, there was essentially no change in the spectrum (Figure 4d) from that obtained with 2 PhSH alone (Figure 4b).

Figure 3 Dependence of degree of cyanide binding to FeMoco on ratio of $[\text{NBu}_4]\text{CN}$ to FeMoco in NMF

E.p.r. spectra were measured at 5 K with a microwave power of 100 mW at 9.435 GHz using 1.9 mT field modulation at 100 kHz. All samples were in NMF and were 2 mM in $\text{Na}_2\text{S}_2\text{O}_4$. (a) Dependence of the $g = 3.82$ signal intensity at 1 mM FeMoco on $[\text{NBu}_4]\text{CN}$ concentration. The fitted curves assume 1.0 (—), 1.5 (—) and 2.0 (---) binding sites and give root mean squares of residuals of 0.055, 0.042 and 0.083 respectively. The fit assuming 1.5 binding sites is the best fit to the data. (b) Job plot with a total concentration ($[\text{NBu}_4]\text{CN} + \text{FeMoco}$) of 1 mM. The fit is to the Job plot function: $y = H([nx + (1-x) + 1/c] - \sqrt{[nx + (1-x) + 1/c]^2 - 4n(x-x^2)})/2n$ where x is the height of the $g = 3.82$ e.p.r. signal (arbitrary units), y is $[\text{NBu}_4]\text{CN}/([\text{NBu}_4]\text{CN} + \text{FeMoco})$, H is a scaling parameter, c is a curvature parameter and n is the number of binding sites. For the best fit, shown by the solid line, n is 1.44 ± 0.02 , where the error is an estimate of the variance of n .

Figure 4 E.p.r. study of relative binding of benzenethiol and cyanide to FeMoco

The samples were 1.0 mM FeMoco (a) alone, plus (b) 2.0 mM benzenethiol, (c) 2.7 mM $[\text{NBu}_4]\text{CN}$, (d) 2.0 mM benzenethiol followed by 2.7 mM $[\text{NBu}_4]\text{CN}$ and (e) 2.7 mM $[\text{NBu}_4]\text{CN}$ followed by 2.0 mM benzenethiol. E.p.r. conditions were as in Figure 3.

Figure 5 M.c.d. spectra of FeMoco and its cyanide adduct

A comparison of the m.c.d. spectra at 5 T and 1.6 K of FeMoco (----) and its cyanide adduct (—). FeMoco (70 μM) was dissolved in NMF/DMF (2:1, v/v). Activity was 285 units/ng-atom of Mo.

M.c.d. measurements

In Figure 5 we compare the m.c.d. spectra of FeMoco and its cyanide adduct. This shows very little change in the form of the spectrum but slight changes in the relative intensities of the bands. The m.c.d. spectra of free FeMoco and of FeMoco bound to benzenethiol and propanethiol are presented in Figure 6. Although the e.p.r. spectra (not shown) demonstrate binding of thiolate, the m.c.d. spectra of thiolate-bound and unbound FeMoco (Figure 6) are remarkably invariant above 450 nm. However, the region to higher energy of 450 nm is less constant. The spectra of Kp1 and FeMoco also differ in this region (Robinson et al., 1984) and so we postulate that this region is particularly sensitive to the FeMoco environment and may

Figure 6 M.c.d. spectra of FeMoco and its thiol adducts at 5 T and 1.6 K

----, FeMoco (70 μM Mo) (activity 285 units/ng-atom of Mo); —, FeMoco + benzenethiol; ····, FeMoco + propanethiol.

Figure 7 M.c.d. magnetization curves of FeMoco-thiol adducts

(a) FeMoco + benzenethiol. Temperatures: $\square$, 1.69 K; $\bullet$, 4.22 K; $\circ$, 9.2 K. Magnetic field varied between 0 and 5 T. Wavelength 387 nm. (b) FeMoco + propanethiol. Temperatures: $\square$, 1.69 K; $\bullet$, 4.22 K; $\circ$, 9.2 K. Magnetic field varied between 0 and 5 T. Wavelength 360 nm. Percentage maximum intensity (%) is plotted against $\beta B/2kt$, where β is the Bohr magneton, B is the magnetic flux density in tesla (T), k is the Boltzmann constant and T is the absolute temperature.

include significant contributions from ligand-to-cluster charge-transfer bands. A band seen near 360 nm in the spectrum of unbound FeMoco is unchanged on binding propanethiol and is red-shifted to 387 nm with an increase in intensity on binding benzenethiol. The benzenethiol result can be explained with

reference to the absorption spectra of molybdenum-iron-sulphur model compounds (Armstrong et al., 1982), where charge-transfer bands from aromatic thiol ligands are shifted to lower energy with respect to aliphatic thiols. The apparent lack of change in the propanethiol-bound adduct is consistent with only one binding site, as is expected from the X-ray model (Kim and Rees, 1992a).

Magnetization curves for the thiolate-bound derivatives are shown in Figure 7. As with the form of the spectrum, magnetization curves of the 520 nm and 800 nm bands (results not shown) remain essentially unchanged from those of FeMoco (with a D value between 4 and 6 cm^{-1}). However, the high-energy bands of the two adducts show very different behaviour. The 360 nm band of FeMoco plus propanethiol (Figure 7b) magnetizes in a similar way to that for FeMoco (Robinson et al., 1984). In contrast, the 387 nm band (Figure 7a) observed with benzenethiol-bound FeMoco magnetizes somewhat differently and cannot therefore have the same assignment.

DISCUSSION

The data presented here show clearly that cyanide binds to the $S = 3/2$ state of FeMoco extracted from the MoFe protein of *K. pneumoniae*. The most direct method of detecting binding is the change in the form of the e.p.r. spectrum of the state with electronic spin $S = 3/2$. The e.p.r. spectrum can only be detected at temperatures less than about 20 K and hence the stoichiometry of ligand binding has been determined only below this temperature. From the variation of e.p.r. intensity as a function of the [cyanide]/[FeMoco] ratio, the stoichiometry of cyanide binding is close to 1.5:1. One interpretation of this is that FeMoco with bound cyanide is not a monomer in NMF solution; for example, it could be a dimer with two terminal and one bridging cyanides per molecule. Alternatively, our preparations contain equal mixtures of FeMoco with one and two cyanide-binding sites. The conclusion of Huang et al. (1993), based on molecular-mass measurements, that FeMoco exists as a dimer in solution in NMF, favours the first of these interpretations.

Conradson et al. (1989) have demonstrated, by means of ^{19}F n.m.r., that the aromatic thiolate ligand, $p\text{-CF}_3\text{C}_6\text{H}_4\text{SH}$, binds at room temperature with a 1:1 stoichiometry to FeMoco from *Azotobacter vinelandii* nitrogenase and further that both cyanide and methylisocyanide (CH_3NC) could bind to the thiolate-FeMoco complex. The binding of cyanide appeared to be weaker at room temperature than in our e.p.r. experiments, as about 8 equivalents of cyanide per FeMoco were required to saturate the binding site or sites. The number of cyanide ligands interacting with the thiolate-FeMoco complex was not determined. The binding was apparently not competitive with thiolate but was an addition reaction. Hence it was concluded that there were separate cyanide (or RNC)- and thiolate-binding sites on FeMoco.

Our e.p.r. data (Figure 4) show that the spectrum obtained with more than one cyanide bound is changed to that of PhS^- -bound FeMoco on addition of PhSH and this process is not reversed by addition of cyanide to PhS^- -bound FeMoco up to a molar ratio of cyanide to FeMoco of 2.7:1. Thus, taken together, the e.p.r. and ^{19}F n.m.r. titrations have shown that, although only one PhS^- per Mo binds to cofactor, both PhS^- and cyanide can bind together.

The following rationalizes these observations: FeMoco clearly has at least two types of cyanide-binding site. If we assume that thiolate ion binds to one of the cyanide sites competitively, then it is the occupancy of this single site, either by cyanide or thiolate, that leads to significant perturbation of the e.p.r. spectrum.

Thiolates must have a considerably higher affinity for this site than does cyanide. E.x.a.f.s. studies have failed to reveal any changes at the molybdenum or iron sites on co-ordination by cyanide but there is evidence that the single thiol binds to an iron site (Harvey et al., 1991). Thus one of the iron atoms of FeMoco seems to have a distinctive reactivity and we postulate that this iron atom also binds cyanide, in the absence of thiolate, resulting in the observed e.p.r. change. The other cyanide-binding site(s) could be an iron or a molybdenum (or even a sulphur) atom. This rationalization is independent of the state of aggregation of FeMoco.

Although the addition of cyanide ligands perturbs the ground electronic state, the spin state remains as $S = 3/2$. There is a small but detectable change in the m.c.d. spectrum, especially in the intensity of the bands between 700 and 850 nm. However, no bands are lost or generated. This is in accord with our finding that the m.c.d. spectrum of FeMoco is remarkably insensitive to its removal from the protein environment. We have discussed above the red shift of the high-energy m.c.d. band on addition of benzenethiol. Our evidence suggests that, with the exception of this band, the optical transitions observed in the m.c.d. spectra of FeMoco and its adducts arise within the $[\text{Fe}_2\text{MoS}_3]$ core and have no contribution from ligands bound to this core.

Our data, combined with those of Conradson et al. (1989), show that cyanide binds to isolated FeMoco at more than one site. One of these sites remains occupied in the thiol complex. Site-directed mutagenesis studies (Kent et al., 1989) have identified a probable cysteine ligand to FeMoco in the α -subunit of Kp1 (Cys-275) and this has been unambiguously shown to be the case for Av1 by X-ray crystallographic analysis (Kim and Rees, 1992a). Therefore we suggest that when FeMoco is bound by this cysteine in the protein, the FeMoco cluster still contains a site at which cyanide can bind under appropriate redox conditions. Kinetic analysis of the reduction of cyanide by nitrogenase (Lowe et al., 1989) demonstrated a long multi-turnover lag before inhibition of H_2 evolution by cyanide and before any cyanide-reduction products could be detected. These data were interpreted in terms of a 'once-only' activation step involving dissociation of a ligand from the active centre. Our observations for free FeMoco indicate that this ligand is unlikely to be Cys-275 as cyanide is unable to replace a thiol. However, it could be His-442 or homocitrate, which have been identified as ligands to Mo in the MoFe protein (Kim and Rees, 1992a).

We thank the SERC for a provision of a CASE award (A.J.M.R.), Carol Gormal for technical support, and the AFRC for support of this work. We also thank Mr. J. Naismith for assistance with the competitive-binding studies and Professor G. D. Watt for communicating data before publication.

REFERENCES

- Armstrong, W. H., Mascharack, P. K. and Holm, R. H. (1982) *Inorg. Chem.* **21**, 1699–1701
- Burgess, B. K. (1990) *Chem. Rev.* **90**, 1377–1406
- Chisnell, J. R., Premakumar, R. and Bishop, P. E. (1988) *J. Bacteriol.* **170**, 27–33
- Clarke, L. and Axley, J. (1955) *Anal. Chem.* **27**, 2000–2006
- Collison, D. and Mabbs, F. E. (1982) *J. Chem. Soc., Dalton Trans.*, 1565–1574
- Conradson, S. D., Burgess, B. K., Vaughn, S. A., Roe, A. L., Hedman, B., Hodgson, K. O. and Holm, R. H. (1989) *J. Biol. Chem.* **264**, 15967–15974
- Eady, R. R. (1986) in *Nitrogen Fixation* (Broughton, W. J. and Pühler, A., eds.), vol. 4, pp. 1–49, Molecular Biology, Clarendon Press, Oxford
- Eady, R. R., Smith, B. E., Cook, K. A. and Postgate, J. R. (1972) *Biochem. J.* **128**, 655–675
- Hardy, R. W. F. and Knight, E. (1967) *Biochim. Biophys. Acta* **139**, 69–90
- Harvey, I., Garner, C. D., Hasnain, S. S., Eady, R. R., Smith, B. E., Richards, R. L. and Gormal, C. A. (1991) in *X-ray Absorption Fine Structure* (Hasnain, S. S., ed.), pp. 171–173, Ellis Horwood, London
- Hawkes, T. R. and Smith, B. E. (1983) *Biochem. J.* **209**, 43–50
- Hawkes, T. R., Lowe, D. J. and Smith, B. E. (1983) *Biochem. J.* **211**, 495–497

- Hawkes, T. R., McLean, P. A. and Smith, B. E. (1984) *Biochem. J.* **217**, 317–321
- Hoover, T. R., Imperial, J., Ludden, P. W. and Shah, V. K. (1989) *Biochemistry* **28**, 2768–2771
- Huang, H. Q., Kofford, M., Simpson, F. B. and Watt, G. D. (1993) *J. Inorg. Biochem.*, in the press
- Kelly, M., Postgate, J. R. and Richards, R. L. (1967) *Biochem. J.* **102**, 1–3
- Kent, H. M., Ioannidis, I., Gomali, C., Smith, B. E. and Buck, M. (1989) *Biochem. J.* **264**, 257–264
- Kim, J. and Rees, D. C. (1992a) *Science* **257**, 1677–1682
- Kim, J. and Rees, D. C. (1992b) *Nature (London)* **360**, 553–560
- Kolthoff, I. M. and Sandell, E. B. (1952) *Textbook of Quantitative Inorganic Analysis*, pp. 458–460, Macmillan Press, London
- Li, J. G., Burgess, B. K. and Corbin, J. C. (1982) *Biochemistry* **21**, 4393–4402
- Lowe, D. J., Eady, R. R. and Thorneley, R. N. F. (1978) *Biochem. J.* **173**, 277–290
- Lowe, D. J., Thorneley, R. N. F. and Smith, B. E. (1985) in *Metalloproteins, Part I, Metal Proteins with Redox Roles* (Harrison, P. M., ed.), pp. 207–249, Macmillan Press, London
- Lowe, D. J., Fisher, K., Thorneley, R. N. F., Vaughn, S. A. and Burgess, B. K. (1989) *Biochemistry* **28**, 8460–8466
- McLean, P. A. and Dixon, R. A. (1981) *Nature (London)* **292**, 655–656
- McLean, P. A., Wink, D. A., Chapman, S. K., Hickman, A. B., McKillop, D. M. and Orme-Johnson, W. H. (1989) *Biochemistry* **28**, 9402–9406
- Newton, W. E., Gheller, S. F., Feldman, B. J., Dunham, W. R. and Schulz, F. A. (1989) *J. Biol. Chem.* **264**, 1924–1927
- Pau, R. N., Mitchenall, L. and Robson, R. L. (1989) *J. Bacteriol.* **171**, 124–129
- Rawlings, J., Shah, V. K., Chisnell, J. R., Brill, W. J., Zimmermann, R., Münck, E. and Orme-Johnson, W. H. (1978) *J. Biol. Chem.* **253**, 1001–1004
- Robinson, A. E., Richards, A. J. M., Thomson, A. J., Hawkes, T. R. and Smith, B. E. (1984) *Biochem. J.* **219**, 495–503
- Robson, R. L., Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M. and Postgate, J. R. (1986) *Nature (London)* **322**, 388–390
- Smith, B. E. (1980) in *Molybdenum Chemistry of Biological Significance* (Newton, W. E. and Otsuka, S., eds.), pp. 179–190, Plenum Press, New York and London
- Smith, B. E. (1983) in *Nitrogen Fixation: The Chemical–Biochemical–Genetic Interface* (Müller, A. and Newton, W. E., eds.), pp. 23–62, Plenum Press, New York and London
- Smith, B. E. and Eady, R. R. (1992) *Eur. J. Biochem.* **205**, 1–15
- Smith, B. E., Lowe, D. J. and Bray, R. C. (1973) *Biochem. J.* **135**, 331–341
- Smith, B. E., Thorneley, R. N. F., Yates, M. G., Eady, R. R. and Postgate, J. R. (1976) *Proc. 1st Int. Symp. Nitrogen Fixation* (Newton, W. E. and Nyman, C. J., eds.), pp. 150–176 Washington State University Press, Pullman
- Smith, B. E., Bishop, P. E., Dixon, R. A., Eady, R. R., Filler, W. A., Lowe, D. J., Richards, A. J. M., Thomson, A. J., Thorneley, R. N. F. and Postgate, J. R. (1985) in *Nitrogen Fixation Research Progress* (Evans, H. J., Bottomley, P. J. and Newton, W. E., eds.), pp. 597–603, Nijhoff, Dordrecht, Boston and Lancaster