421

Iron K-edge X-ray absorption spectroscopy of the iron–molybdenum cofactor of nitrogenase from *Klebsiella pneumoniae*

Judith M. ARBER,* Annette C. FLOOD,* C. David GARNER,*§ Carol A. GORMAL,† S. Samar HASNAIN‡ and Barry E. SMITH†
*Department of Chemistry, University of Manchester, Manchester M13 9PL, U.K., †AFRC, Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, U.K., and ‡SERC, Daresbury Laboratory, Daresbury, Warrington WA4 4AD, Cheshire, U.K.

Iron K-edge X-ray absorption data for the iron–molybdenum cofactor ('FeMoco') from *Klebsiella pneumoniae* reported here provide the first evidence for long-range structural order in the cofactor [Fe...Fe(Mo) = 0.368 nm in addition to Fe...S = 0.22 nm and Fe...Fe(Mo) = 0.27 nm] and, in contrast with previously published data [Antonio, Teo, Orme-Johnson, Nelson, Groh, Lindahl, Kauzlarich & Averill (1982) J. Am. Chem. Soc. **104**, 4703–4705], indicate that most of the iron centres are not co-ordinated to light (oxygen, nitrogen) atoms. This demonstrates that presently available chemical models for FeMoco are inadequate.

INTRODUCTION

The molybdenum-containing nitrogenases from all diazotrophic bacteria studied consist of an iron protein and a molybdenum–iron (MoFe) protein. The latter contains up to two molybdenum and about 30 iron and acid-labile sulphur atoms which are grouped into two FeMoco (iron–molybdenum cofactor) centres, probably four [4Fe–4S] 'P' clusters, and perhaps two 'S' iron atoms of unknown structure [see Smith (1983) and Lowe *et al.* (1985)]. The FeMoco centres can be extracted into *N*-methylformamide (NMF) from the MoFe protein. Extracted FeMoco samples from all species seem to be identical and can activate the MoFe protein polypeptides in mutants of *Klebsiella pneumoniae* or *Azotobacter vinelandii* that are unable to synthesize FeMoco.

FeMoco is believed to be the site of N_2-binding in molybdenum-containing nitrogenases (Hawkes *et al.*, 1984). It has therefore been extensively studied by a range of biochemical and spectroscopic techniques (reviewed by Lowe *et al.*, 1985), and these results, together with analytical data, suggest that FeMoco contains one molybdenum, six to eight iron and four to nine sulphur atoms and is anionic. The iron environments are not all equivalent; ^{57}Fe Mössbauer data indicate the presence of six paramagnetic iron centres of two different types (Hunyh *et al.*, 1979, 1980), and ENDOR results suggest at least five distinct iron sites (Hoffmann *et al.*, 1982*a,b*; Venters *et al.*, 1986).

Most X-ray-absorption studies of nitrogenase have focused on the environment of molybdenum in the MoFe protein and in FeMoco extracted into NMF, and the interpretations of the extended X-ray-absorption-fine structure (e.x.a.f.s.) data imply that molybdenum has three or four sulphur atoms at 0.24 nm, two to four iron atoms at 0.27 nm and, possibly, one or two oxygen or

nitrogen atoms at 0.22 nm in its immediate environment (Cramer *et al.*, 1978*a,b*; Eidsness *et al.*, 1986; Flank *et al.*, 1986; Flood, 1986). A recent analysis of molybdenum K-edge X-ray absorption near edge structure (x.a.n.e.s.) data suggests that molybdenum is co-ordinated to three oxygen and three sulphur ligands (Conradson *et al.*, 1985). Previously reported iron K-edge e.x.a.f.s. data (Antonio *et al.*, 1982) were simulated by back-scattering from 3.4 ± 1.6 sulphur atoms at 0.225(2) nm, 2.3 ± 0.9 iron atoms at 0.266(3) nm, 0.4 ± 0.1 molybdenum atoms at 0.276(3) nm and 1.2 ± 1.0 oxygen/nitrogen atoms at 0.181(7) nm. Thus e.x.a.f.s. data from both metal K-edges support the idea of an Fe–Mo–S cluster similar to, but not identical with, some of the synthetic Fe–Mo–S clusters (see, for example, Holm, 1981). The present paper reports new iron K-absorption edge data for native FeMoco that show clear differences from those published by Antonio *et al.* (1982).

MATERIALS AND METHODS

Samples of FeMoco were prepared from *Klebsiella pneumoniae* and isolated and assayed as previously reported (Smith, 1980), the cofactor being extracted into NMF and concentrated by evaporation to a final iron concentration of 7.5–9.5 mM. Samples were stored in liquid nitrogen after anaerobic loading into sealed cells. Sample activity was assayed before and after irradiation, and e.p.r. spectra were measured after irradiation. [A unit of FeMoco activity ≡ 1 nmol of acetylene reduced/min after incorporation into the inactive MoFe protein of nitrogenase, Nif B⁻ Kp1 (Hawkes & Smith, 1983).] The extensive handling in the recovery of the oxygen-sensitive FeMoco samples would generally be expected to result in some loss in activity and, since an average of 80 %

Abbreviations used: e.x.a.f.s., extended X-ray absorption fine structure; FeMoco, iron–molybdenum cofactor; NMF, *N*-methylformamide; x.a.n.e.s., X-ray absorption near-edge structure; ENDOR, electron nuclear double resonance.
    § To whom correspondence and reprint requests should be addressed.

activity was recovered after irradiation and measurement of e.p.r. spectra, we conclude that the 20 % damage can be attributed to handling after irradiation rather than to radiation damage. Aliquots of all samples were evaporated to dryness in boiling tubes and then digested with HNO_3 and H_2SO_4 before measurement of their iron and molybdenum contents by the methods of Eady et al. (1972).

X-ray absorption spectra were recorded in the fluorescence mode on e.x.a.f.s. stations 8.1 and 7.1 at the Daresbury Synchrotron Radiation Source operating at 2 GeV and an average current of 150 mA; about seven scans were recorded for each sample and the data averaged. On station 8.1, a slit-less double crystal Si(111) monochromator was employed, thus minimizing har-

monic contamination (van der Hoek et al., 1986), the novel design of the monochromator (Dobson et al., 1986) allowing high-resolution X-ray absorption edge and x.a.n.e.s. data to be collected. An average sample temperature of 80 K was maintained during measurement by use of a liquid-nitrogen cryostat. On station 7.1, e.x.a.f.s. data were collected using a channel-cut Si(111) monochromator with a double focusing mirror and a liquid-helium cryostat (Oxford Instruments; CF1208), the average sample temperature in these experiments being 25 K. Four samples were studied, and no significant differences were observed between the data collected under the different experimental conditions. Data analysis was accomplished via the single-scattering spherical-wave method for e.x.a.f.s. and phase shifts were derived from 'ab initio' calculations as described previously (Lee & Pendry, 1975; Perutz et al., 1982; Gurman et al., 1984). Iron K-edge e.x.a.f.s. data for several model compounds were analysed in order to define the accuracy of the phase shifts (Flood, 1986).

RESULTS AND DISCUSSION

Fig. 1 shows the iron K-edge e.x.a.f.s. and Fourier transform of FeMoco together with a theoretical simulation using the parameters listed in Table 1. There is clear evidence for back-scattering from sulphur atoms at 0.22 nm and from a shell of iron atoms at about 0.27 nm. Other evidence (Lowe et al., 1985) demonstrates that molybdenum is an integral part of FeMoco and, consistent with the molybdenum K-edge e.x.a.f.s. results (Cramer et al., 1978a,b; Eidsness et al., 1986; Flank et al., 1986; Flood, 1986), we have included (Table 1) back-scattering from molybdenum for at least some of the iron atoms. The contribution from molybdenum back-scattering is nearly out of phase with that of iron in the low k region of the spectrum, and the effect of this smaller molybdenum back-scattering contribution can be partially compensated for by a slightly shorter Fe...Fe distance (about 0.262 nm). However, this results in a
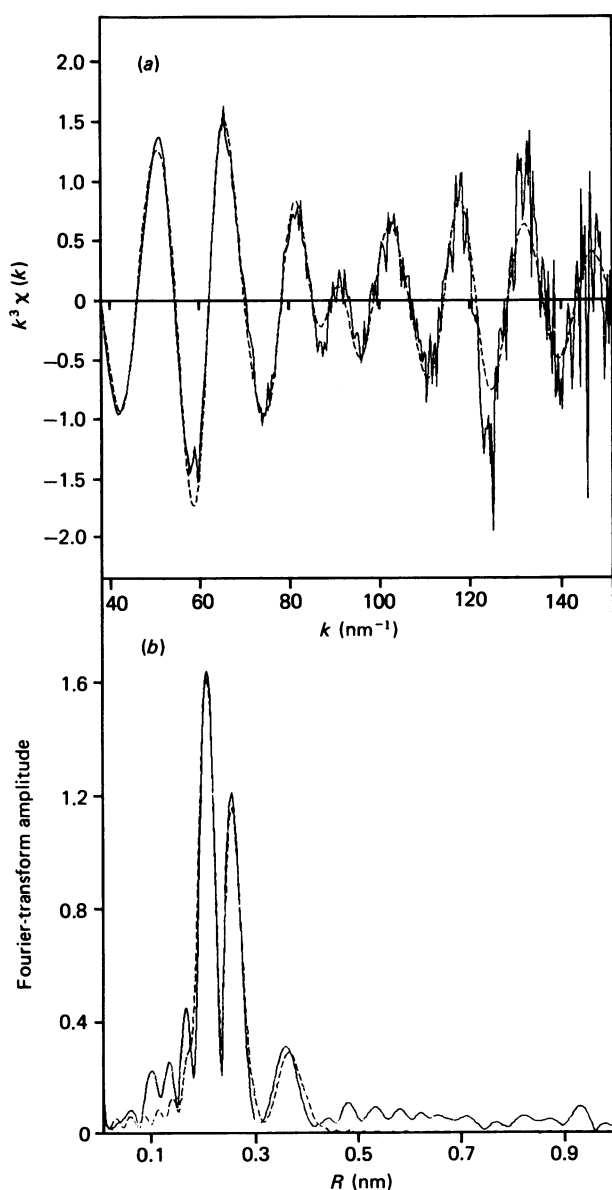

Fig. 1. (a) Fe K-edge e.x.a.f.s. (χk^3) and (b) Fourier transform of FeMoco with final simulation based on the parameters given in Table 1

The continuous line represents experimental data; the broken line the theoretical simulation.

Table 1. Parameters used to simulate the e.x.a.f.s. associated with the iron K-edge of FeMoco

E_0 (the photoionization threshold) $= 19.70$ eV; for each shell N is the co-ordination number, R the distance and $2\sigma^2$ the Debye–Waller parameter, which includes both static and thermal disorder. The least-squares refinement procedure used here provides an estimate of the fitting errors for the refined parameters. In addition, the errors can be estimated by examining the quality of the simulation by eye when changing the parameters beyond the errors indicated by least-squares refinement. The errors obtained by the second method are larger ($\sim N \pm 10\%$, $R \pm 1\%$ and $2\sigma^2 \pm 10\%$). In view of the interfering nature of the iron and molybdenum back-scattering contributions at ~ 0.27 nm (see the text), parameters for this shell are expected to be in error by $\sim N \pm 20\%$, $R \pm 3\%$ and $2\sigma^2 \pm 20\%$.

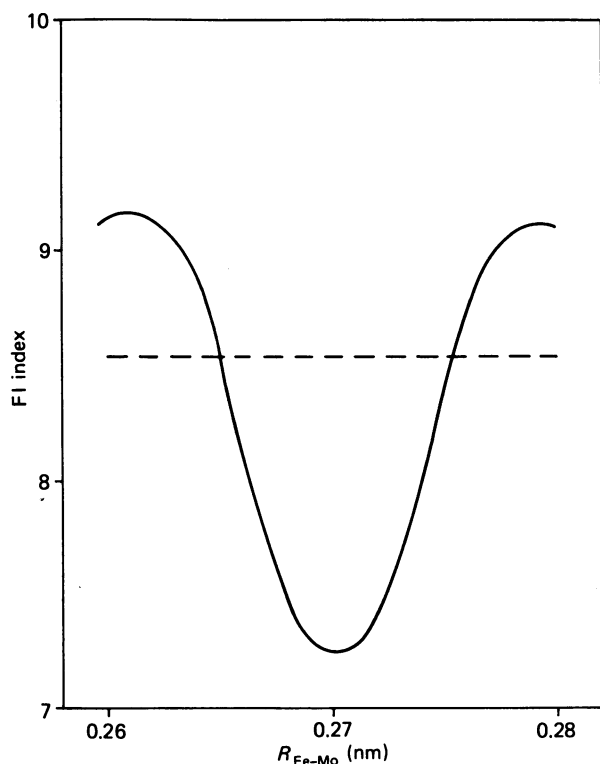| Atom	N	R(nm)	$2\sigma^2$(nm^2)
S	3.0	0.220	0.00010
Fe	2.2	0.264	0.00013
Mo	0.8	0.270	0.00014
Fe	1.3	0.368	0.00014

Fig. 2. Variation of least-squares fit index (FI) for Fe K-edge e.x.a.f.s. simulations of FeMoco as a function of Fe–Mo distance

The broken line is the value of the fit index when the second shell Mo is omitted from the simulation. FI is defined as the weighted sum of experimental-minus-theoretical data points:

$$FI = [X(k)_{experiment} - X(k)_{theory}]/N$$

where N is the number of data points in the experimental spectrum.

significant deterioration in the quality of the simulation, notably in the reproduction of the phase below $\sim k = 110 \, nm^{-1} \, (11 Å^{-1})$. The necessity for the inclusion of molybdenum back-scattering was also assessed by plotting the fit index (Binsted et al., 1987) as a function of the distance of molybdenum from the absorbing atom (Fig. 2), the results demonstrating the decrease in fit index when molybdenum backscattering is included in the simulation. These conclusions imply that FeMoco

$$Fe \overset{\displaystyle S}{\underset{\displaystyle S}{\diagup\diagdown}} Fe(Mo)$$
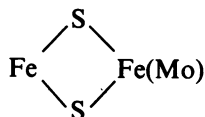
contains rhombs of metal–sulphur atoms, consistent with the work of Antonio et al. (1982) and as found in a wide variety of Fe–S and Fe–Mo–S clusters (Holm, 1981). The distances involved indicate an average Fe–S–Fe(Mo) angle of $\sim 74\,°$ (scattering angle of $\sim 106°$); we note that multiple scattering effects are not present at scattering angles of more than 90° (Gurman et al., 1986).

However, there are two major differences between the e.x.a.f.s. data shown here and those of Antonio et al.

(1982). The first is the observation of a shell of atoms, beyond the $FeS_2Fe(Mo)$ rhombs implied by the peak in the Fourier transform at $\sim 0.37 \, nm$ (Fig. 1). The form of this contribution (shown by the difference spectrum, Fig. 3) indicates heavy-atom back-scattering and, given the known chemical components of the cofactor (see above), and the characteristics of this back-scattering, either iron or molybdenum would seem an appropriate choice. Simulations (Fig. 3; note caption) suggest that iron back-scattering makes the dominant contribution. Nevertheless, the fact that we are considering an average iron environment in the e.x.a.f.s. may mean that there is a certain amount of both types of metal back-scattering present. The observation of this metal back-scattering suggests that FeMoco possesses an ordered Fe–Mo–S framework, i.e. the various back-scattered contributions are coherent. An average Fe–S–Fe angle of $\sim 113°$ (scattering angle of $\sim 67°$) is implied within the longer unit, and thus multiple scattering contributions are negligible, producing no change in the distances reported here and no significant change in the fit index.

The presence of a long metal–metal interaction suggests that the $[Fe_6S_6X_6]^{3-}$ (X = halide) core synthesized by Kanatzidis et al. (1985), i.e. with two distinct iron–iron distances of 0.276 and 0.379 nm and an average iron–sulphur distance of 0.228 nm [Fe–S–Fe angles, 74.7 (2)° and 113.2 (3)°], might be an appropriate structural model for the type of metal–sulphur cluster in FeMoco. However, we note that the distances reported here for FeMoco are all significantly shorter and that e.x.a.f.s. data for $[Fe_6S_6Cl_6]^{3-}$ do not show evidence for any long Fe–Fe back-scattering contribution. Furthermore, an inspection of the dimensions of the Fe–S frameworks of crystallographically characterized chemical systems suggested as possible models for FeMoco reveals that each is deficient in at least one respect, as compared with the results of the iron K-edge e.x.a.f.s. described here.

The second difference between the data presented in Fig. 1 and those of Antonio et al. (1982) is the absence of any distinct peak in the Fourier transform which could be attributed to a light atom. It is possible to include a small back-scattering contribution (~ 0.5 atom) from oxygen or nitrogen in the data-fitting procedure, but, on least-squares refinement, the resultant Fe–O,N distance refines to a value of between 0.159 (5) nm and 0.213 (11) nm, depending on the initial value chosen for refinement and does not produce any significant decrease in the fit index. This situation is in clear contrast with the iron K-edge e.x.a.f.s. data for $[Fe_2S_2(OPh)_4]^{2-}$ (Antonio et al., 1982) (OPh is phenoxide), which shows a distinct peak in the Fourier transform attributable to oxygen back-scattering. Therefore, although it is not possible to exclude completely light-atom co-ordination to iron in FeMoco, the present study provides no clear evidence for such bonding. This situation mirrors that for the molybdenum K-edge e.x.a.f.s. of the MoFe protein, where inclusion of a Mo–O,N component at 0.182 nm, as suggested by x.a.n.e.s. data (Conradson et al., 1985), is not essential for the reproduction of the main features of the e.x.a.f.s. but does produce a small improvement in the quality of the fit (Eidsness et al., 1986; Flood, 1986).

One of the samples we studied did show evidence for back-scattering from oxygen (carbon or nitrogen) at $\sim 0.18 \, nm$, as reported by Antonio et al. (1982). This sample had a higher iron/molybdenum ratio ($9 \pm 1 : 1$)
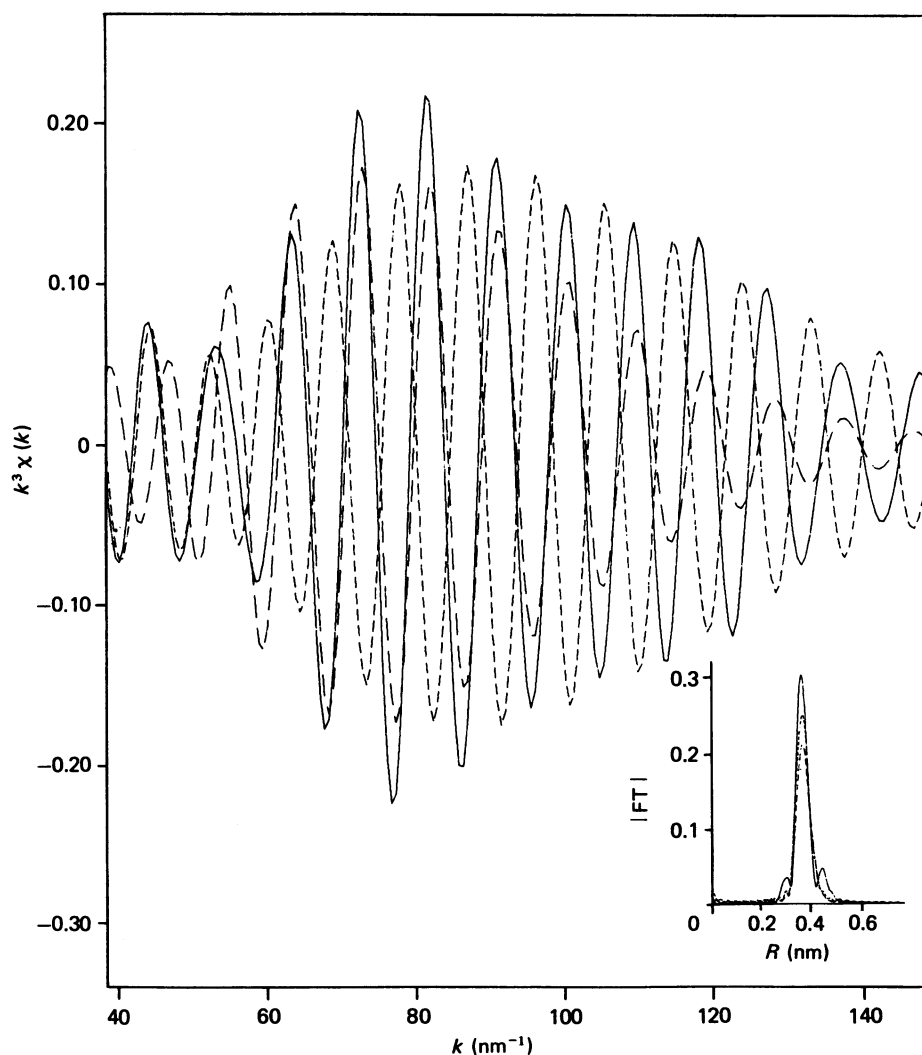
Fig. 3. Difference spectrum from Fe K-edge e.x.a.f.s. of FeMoco

The continuous line shows the experimental spectrum (χk^3) after subtraction of the best two-shell fit and subsequent Fourier filtering with a window of 0.260–0.420 nm. The broken lines represent theoretical simulations of one molybdenum atom at 0.362 nm (----; fit index = 2.84) and one iron atom at 0.366 nm (-----; fit index = 0.29). In both simulations $2\sigma^2 = 0.00014$ nm^2 and $E_0 = 19.70$ eV.
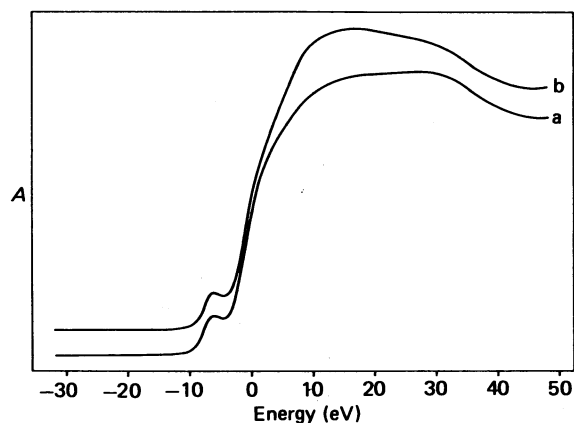

Fig. 4. Fe K-edge and x.a.n.e.s. of (a) FeMoco (activity 118 units/ng-atom of molybdenum) and (b) FeMoco sample as in a, but after warming up etc. (see the text); only 40% of sample activity was recovered

than those of other samples (with ratios of 6.6–6.8:1) that did not show evidence for oxygen back-scattering. The activity of this sample was 62.5 units/ng-atom of molybdenum, i.e. of significantly higher activity than the lowest-activity sample we have studied (36 units/ng-atom of molybdenum; iron/molybdenum ratio, 6.70), the latter sample showing no appreciable light-atom back-scattering component. Therefore it seems probable that the strong oxygen back-scattering at ~ 0.18 nm is associated with extraneous iron, which would be expected to be co-ordinated by oxygen or nitrogen ligands in NMF solution.

The X-ray absorption spectra shown in Figs. 1 and 3 were recorded by using a sample of FeMoco with an activity of 190 units/ng-atom of molybdenum. A range of samples with activities of between 36 and 190 units/ng-atom of molybdenum has been studied, and the parameters used in the simulation of each set of e.x.a.f.s. data are in agreement with the parameters in Table 1 within the errors given there. Furthermore, after e.x.a.f.s.

analysis, one sample was allowed to warm up to room temperature aerobically for a few minutes, and then refrozen in liquid nitrogen and the X-ray absorption spectrum re-measured. No change was observed in the e.x.a.f.s.. Fig. 4 shows the iron K-absorption edge and x.a.n.e.s. data for this sample before and after this treatment. Both spectra exhibit a weak pre-edge feature and an edge inflection of comparable intensities, implying the same (average) electronic structure for the iron sites; the marked differences in the x.a.n.e.s. of the two samples (which is sensitive to multiple scattering contributions) suggest differences in the detailed geometry of the iron sites in these systems. Since the e.x.a.f.s. of each are virtually identical, we conclude that the geometrical changes are primarily angular (rather than radial). Subsequent measurement showed that the sample had lost about 60% of its activity. The change in colour of FeMoco solutions from brown to red on prolonged exposure to oxygen presumably is due, at least in part, to the formation of oxothiomolybdate species (Zumft, 1978) on disintegration of the cluster. However, the data presented here indicate that loss of FeMoco activity can occur through relatively minor and subtle changes in structure at the iron atoms of the cluster structure.

We conclude, on the basis of the iron K-edge e.x.a.f.s. data presented herein, that native FeMoco possesses a more extended structure than formerly observed, and we have found no evidence for the co-ordination of iron by light (O, N) atoms. Thus presently available chemical models do not accurately represent the organization of the cofactor cluster, and more synthetic work is necessary. In addition, the data presented imply that the initial phases of loss of activity in FeMoco are not associated with any major changes in the average iron environment. The present results are also relevant to the recently isolated vanadium-containing nitrogenase of *Azotobacter chroococcum* (Robson *et al.*, 1986), since it is now known that vanadium is part of a corresponding cofactor (Smith *et al.*, 1988), and e.x.a.f.s. data show (Arber *et al.*, 1987) that the vanadium–iron protein is in a similar environment to molybdenum in FeMoco and the MoFe protein.

REFERENCES

Antonio, M. R., Teo, B.-K., Orme-Johnson, W. H., Nelson, M. J., Groh, S. E., Lindahl, P. A., Kauzlarich, S. M. & Averill, B. A. (1982) J. Am. Chem. Soc. **104**, 4703–4705

Arber, J. M., Dobson, B. R., Eady, R. R., Stevens, P., Hasnain, S. S., Garner, C. D. & Smith, B. E. (1987) Nature (London) **325**, 372–374

Binsted, N., Cook, S. L., Evans, J., Greaves, G. N. & Price, R. J. (1987) J. Am. Chem. Soc. **109**, 3669–3676

Conradson, S. D., Burgess, B. K., Newton, W. E., Hodgson, K. O., McDonald, J. W., Rubinson, J. F., Gheller, S. F., Mortenson, L. E., Adams, M. W. W., Mascharak, P. K., Armstrong, W. A. & Holm, R. H. (1985) J. Am. Chem. Soc. **107**, 7935–7940

Cramer, S. P., Hodgson, K. O., Gillum, W. O. & Mortenson, L. E. (1978a) J. Am. Chem. Soc. **100**, 3398–3407

Cramer, S. P., Gillum, W. O., Hodgson, K. O., Mortenson, L. E., Stiefel, E. I., Chisnell, J. R., Brill, W. J. & Shah, V. K. (1978b) J. Am. Chem. Soc. **100**, 3814–3819

Dobson, B. R., Hasnain, S. S., Hart, M., van der Hoek, M. J. & van Zuylen, P. (1986) J. Phys. Colloq. (Orsay, Fr.) **47**, C8, 121–125

Eady, R. R., Smith, B. E., Cook, K. A. & Postgate, J. R. (1972) Biochem. J. **128**, 655–675

Eidsness, M. K., Flank, A. M., Smith, B. E., Flood, A. C., Garner, C. D. & Cramer, S. P. (1986) J. Am. Chem. Soc. **108**, 2746–2747

Flank, A. M., Weininger, M., Mortenson, L. E. & Cramer, S. P. (1986) J. Am. Chem. Soc. **108**, 1049–1055

Flood, A. C. (1986) Ph.D. Thesis, University of Manchester

Gurman, S. J., Binsted, N. & Ross, I. (1984) J. Phys. C **17**, 143–151

Gurman, S. J., Binsted, N. & Ross, I. (1986) J. Phys. C **19**, 1845–1861

Hawkes, T. R. & Smith, B. E. (1983) Biochem. J. **209**, 43–50

Hawkes, T. R., McLean, P. A. & Smith, B. E. (1984) Biochem. J. **217**, 317–321

Hoffman, B. M., Roberts, J. E. & Orme-Johnson, W. H. (1982a) J. Am. Chem. Soc. **104**, 860–862

Hoffman, B. M., Venters, R. A., Roberts, J. E., Nelson, M. & Orme-Johnson, W. H. (1982b) J. Am. Chem. Soc. **104**, 4711–4712

Holm, R. H. (1981) Chem. Soc. Rev. **10**, 455–490

Hunyh, B. H., Munck, E. & Orme-Johnson, W. H. (1979) Biochim. Biophys. Acta **576**, 192–203

Hunyh, B. H., Henzl, M. T., Christner, J. A., Zimmerman, R., Orme-Johnson, W. H. & Munck, E. (1980) Biochim. Biophys. Acta **623**, 124–138

Kanatzidis, M. G., Hagen, W. R., Dunham, W. R., Lester, R. K. & Coucouvanis, D. (1985) J. Am. Chem. Soc. **107**, 953–961

Lee, P. A. & Pendry, J. B. (1974) Phys. Rev. B **11**, 2795–2811

Lowe, D. J., Thorneley, R. N. F. & Smith, B. E. (1985) in Metalloproteins, Part 1: Metal Proteins with Redox Roles (Harrison, P. M., ed.), pp. 207–249, Verlag Chemie, Weinheim

Perutz, M. F., Hasnain, S. S., Duke, P. J., Sessler, J. L. & Hahn, J. E. (1982) Nature (London) **295**, 535–538

Robson, R. L., Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M. & Postgate, J. R. (1986) Nature (London) **322**, 388–390

Smith, B. E. (1980) in Molybdenum·Chemistry of Biological Significance (Newton, W. E. & Otsuka, S., eds), pp. 179–190, Plenum Press, New York

Smith, B. E. (1983) in Nitrogen Fixation: The Chemical, Biochemical and Genetical Interfaces (Muller, A. & Newton, W. E., eds.), pp. 23–62, Plenum Press, New York

Smith, B. E., Eady, R. R., Lowe, D. J. & Gormal, C. A. (1988) Biochem. J. **250**, 299–302

van der Hoek, M. J., Werner, W., van Zuylen, P., Dobson, B. R., Hasnain, S. S., Worgan, J. S. & Luickx, G. (1986) Nucl. Instrum. Methods A**246**, 380–384

Venters, R. A., Nelson, M. J., McLean, P. A., True, A. E., Levy, M. A., Hoffman, B. M. & Orme-Johnson, W. H. (1986) J. Am. Chem. Soc. **108**, 3487–3498

Zumft, W. J. (1978) Eur. J. Biochem. **91**, 345–350