Synergic Binding of Carbon Monoxide and Cyanide to the FeMo Cofactor of Nitrogenase: Relic Chemistry of an Ancient Enzyme?

Christopher J. Pickett,*[a] Kylie A. Vincent,[b] Saad K. Ibrahim,[a] Carol A. Gormal,[a] Barry E. Smith,[a] Shirley A. Fairhurst,[a] and Stephen P. Best*[b]

Abstract: The first electrochemical and infra-red data on the binding of cyanide to the isolated iron–molybdenum cofactor of nitrogenase, FeMoco, is described. It is shown that cyanide stabilises a hitherto unrecognised, low-spin, EPR-active ($S = {}^1/_2$), superoxidised form of FeMoco, and we provide the first evidence that carbon monoxide and cyanide bind synergically to the oxidised and semireduced states of the isolated cofactor, states which are unreactive to carbon monoxide alone.

Keywords: carbon monoxide · cofactors · cyanides · electrochemistry · EPR spectroscopy · IR spectroscopy · nitrogenases

Introduction

The small molecules cyanide and carbon monoxide play a significant role as essential ligands at the active site of the iron-only and nickel–iron hydrogenases; the hydrogen-metabolising metallo–sulfur enzymes found widespread amongst the Archaea and other microrganisms.[1,2] Nitrogenase, the enzyme system that reduces dinitrogen to ammonia, also catalyses hydrogen production from protons, and this occurs at the iron–molybdenum cluster of the enzyme (FeMoco).[3–5] Intriguingly, under certain conditions, this metallo–sulfur cluster also coordinates carbon monoxide and cyanide.[3,6] Alone, cyanide suppresses proton and substrate reduction, but in the co-presence of carbon monoxide the production of dihydrogen is restored.[7]

Interactions of cyanide, carbon monoxide and other small molecules with nitrogenase may "simply" reflect the extraordinary reactivity of sites on the FeMoco cluster, which must bind, activate and reduce the rather inert molecule, dinitrogen. However, it has been argued, on the basis of phylogenetic evidence, that the modern nitrogen-fixing apparatus may have evolved from ancient cyanide-metabolising or detoxifying iron–sulfur systems, with the nitrogen-reducing activity "bolted on" later by means of molybdenum incorporation in response to the depletion of fixed nitrogen in the biosphere.[8] Thus, interactions of the FeMoco cluster in the enzyme with cyanide, carbon monoxide, ethyne and so forth at the iron–sulfur core may reflect "relic chemistry", whereas their interactions at Mo may reflect the intrinsic high reactivity of a dinitrogen binding and activating site. Some support for such dual chemistry at FeMoco, centred either at Mo or the Fe cluster core, comes from our recent spectroelectrochemical work with FeMoco isolated from the protein.[6,9] Two independent interactions with CO are observed, and we have associated these with reduction at essentially insulated Mo and Fe core redox sites.[6] Evidence for cyanide binding at Mo and/or Fe sites of the isolated cofactor has been obtained by others in a range of kinetic,[10] NMR,[11] EPR,[12,13] and Extended X-ray Absorption Fine Structure (EXAFS)[12] studies.

In this paper we report: (1) The first electrochemical and infra-red data for cyanide bound to FeMoco; (2) that cyanide stabilises a hitherto unrecognised, low-spin, EPR-active, superoxidised form of the isolated cofactor and (3) the first evidence that carbon monoxide and cyanide bind synergically to the oxidised and semireduced states of the isolated cofactor—states that are unreactive to carbon monoxide alone.[9]

Results and Discussion

The FeMo cofactor was extracted, purified and concentrated from the iron–molybdenum nitrogenase protein of *Klebsiella pneumoniae* into *N*-methylformamide (NMF) containing dithionite, phosphate buffer and approximately 2% water (see Experimental Section). Upon isolation, the cofactor is in the

[a] Prof. C. J. Pickett, Dr. S. K. Ibrahim, C. A. Gormal, Prof. B. E. Smith, Dr. S. A. Fairhurst
Department of Biological Chemistry, John Innes Centre
Norwich NR4 7UH (UK)
Fax: (+44) 1603-450-018
E-mail: chris.pickett@bbsrc.ac.uk

[b] K. A. Vincent, Dr. S. P. Best
School of Chemistry, University of Melbourne
Victoria 3010 (Australia)
Fax: (+61) 393-475-180
E-mail: spbest@unimelb.edu.au

DOI: 10.1002/chem.200400382

EPR-active ($S=^3/_2$), semireduced form, FeMoco^{semired}.[14] In contrast, under an inert atmosphere, dithionite is slowly consumed and the cofactor is (reversibly) oxidised to the EPR-silent FeMoco^{ox} form, as described by Schultz and co-workers.[15] This oxidised form or its thiophenolate derivative, which has PhS^- ligated to the terminal Fe atom, provides the starting point for the studies described herein (Figure 1).

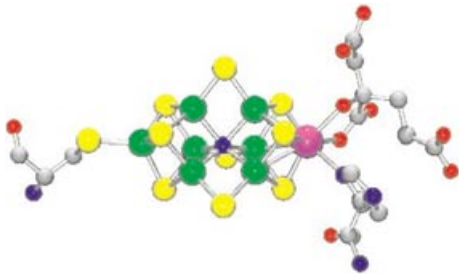


Figure 1. Structure of FeMoco, adapted from reference [5] (Fe, S, N, O, C and Mo atoms are represented as green, yellow, blue, red, grey and purple spheres, respectively).

The addition of cyanide to solutions of either the native, oxidised cofactor FeMoco^{ox}, or its thiophenolate-modified form FeMoco(SPh)^{ox} in NMF, results in dramatic changes in their voltammograms. Figure 2a shows the change in the cyclic voltammetry of FeMoco(SPh)^{ox} with increasing cyanide concentration. The primary reduction couple FeMoco(SPh)$^{ox/semired}$ (process **I**) at $E^{\circ\prime}=-0.55$ V is suppressed and two new processes appear with $E^{\circ\prime}=-0.91$ V (process **R**) and $E^{\circ\prime}=-0.45$ V (process **O**).[16] At fast scan rates, both processes are reversible and the FeMoco(SPh)$^{ox/semired/red}$ couples (processes **I** and **II**) are nearly fully suppressed, leaving a well-defined, three-membered electron-transfer series (Figure 2b). At slower scan rates, process **R** is only partially reversible (Figure 2, inset), and this is associated with the partial recovery of processes **I** and **II** of FeMoco(SPh). This shows that slow dissociation of cyanide ($t_{1/2}\approx 10$ s) occurs at reduced redox levels of the cofactor, and that the binding of cyanide to FeMoco(SPh)^{ox} is tighter than to FeMoco(SPh)^{semired}.

Similar changes to the voltammetry were observed when cyanide was added to the native NMF-isolated cofactor, FeMoco^{ox} (data not shown).[6]

We have observed a process associated with the reduction of FeMoco(SPh)^{red}, which we have previously argued is Mo-centred reduction, corresponding to process **III** in Figure 2 (inset, solid line).[6] Addition of CN^- has no effect on the potential of this reduction process (Figure 2 inset, dashed line). This would suggest that either cyanide does not bind to Mo or that it replaces an anionic ligand from the Mo atom with negligible effect on the electronic distribution. We discuss this further below.

Anionic ligands, such as cyanide, which are strong σ donors stabilise metals in a higher oxidation state, that is, they shift E° in a negative direction. This would suggest that process **R** is more likely to be associated with the oxidised/semireduced levels of the cofactor and that process **O** accesses a new, superoxidised level, FeMoco(CN)^{superox}. This assignment of the redox levels involved in the two processes is confirmed by EPR spectroscopy. The semireduced level of FeMoco is associated with a well-defined $S=^3/_2$ EPR signal (Figure 3a), whereas the oxidised level is EPR silent.[15] Figure 3b shows the EPR spectrum recorded after addition of excess $[Et_4N]CN$ to a solution of FeMoco^{ox}. Oxidation of this solution by bulk electrolysis at −0.35 V at a platinum electrode results in an $S=^1/_2$ species with a well-defined, rhombic EPR signal (Figure 3c; $g_x=2.268$, $g_y=1.972$ and $g_z=1.868$ obtained from simulation of the spectrum), consistent with a low-spin electron configuration. Both higher oxidation levels and strong-field ligands will favour low-spin states. Iron–sulfur systems with multiple Fe^{II} centres (as in the $[4Fe{-}4S]^{3+}$ cluster of oxidised high-potential iron–sulfur proteins (HiPIPs), such as that from *Allochromatium vinosum*), also show an $S=^1/_2$ spin system.[17] The absence of ^{95}Mo hyperfine lines in the spectrum is concordant with unpaired spin density residing on the iron core. Thus, process **O** can be assigned to a FeMoco(CN)$^{superox/ox}$ couple, in which FeMoco(CN)^{superox} is at an oxidation level 2*e* higher than FeMoco^{semired}.

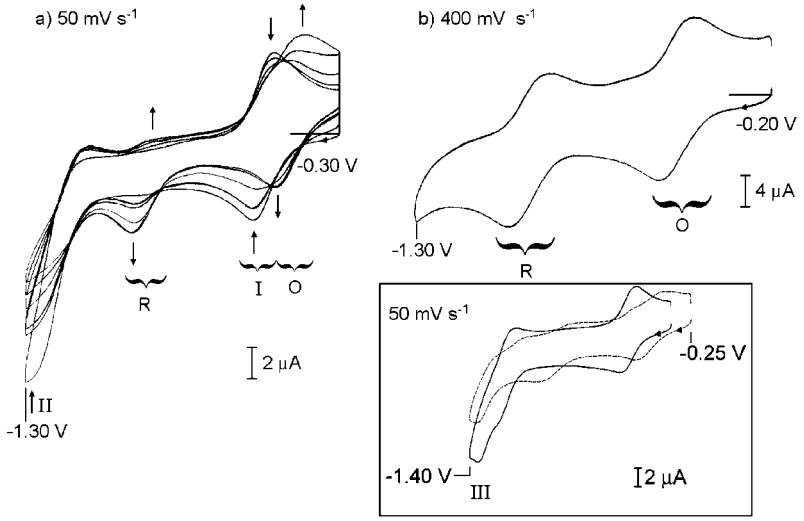


Figure 2. Cyclic voltammograms (current vs potential) for an NMF solution of FeMoco(SPh)^{ox} recorded at a) 50 mV s^{-1} during titration with cyanide: 0, 5, 10, 15 and 20 molar equivalents of $[Et_4N]CN$ (arrows indicate the direction of current change with increasing cyanide); and b) 400 mV s^{-1} after addition of 28 molar equivalents of $[Et_4N]CN$. Processes **I** and **II** are associated with FeMoco(SPh), whereas processes **O** and **R** are associated with the cyanide-modified cofactor. Inset, recorded at 50 mV s^{-1} over a slightly wider potential range with no cyanide (solid line) and after addition of 28 molar equivalents of $[Et_4N]CN$ (dashed line). At the commencement potential for these voltammograms FeMoco(SPh) is present at the oxidised level, while the cyanide-ligated cofactor is predominantly at the superoxidised level.

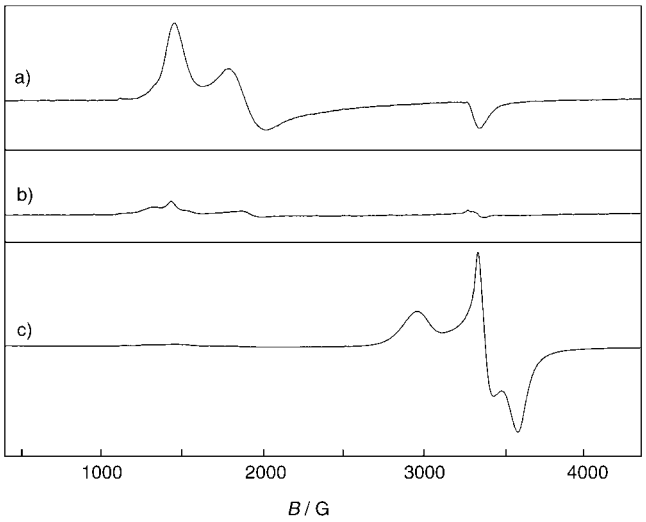


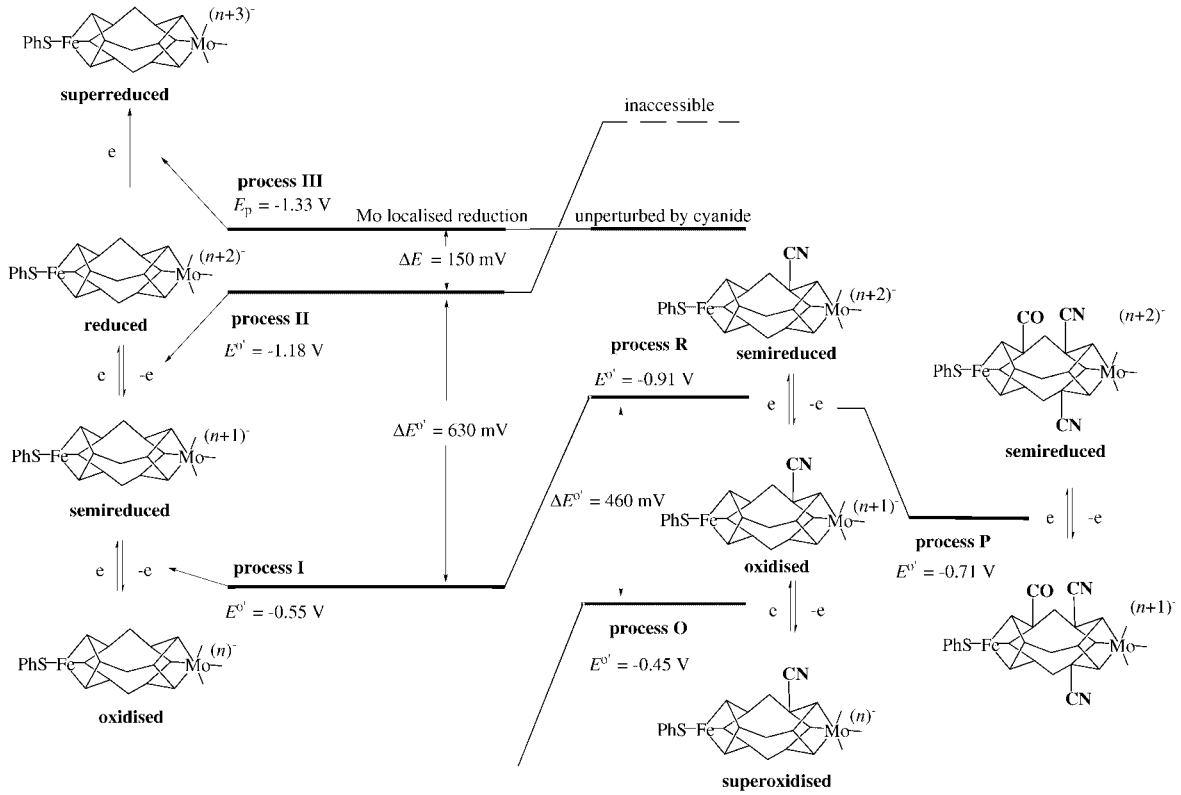
Figure 3. EPR spectra recorded at 10 K, 100 mW, for NMF solutions of FeMoco: a) FeMoco^{semired} prepared by chemical reduction of FeMoco^{ox} with sodium dithionite; b) FeMoco^{ox} after addition of an excess of $[Et_4N]CN$; and c) after oxidative electrolysis of the solution in b) at −0.35 V, showing that cyanide binding allows access to a new, EPR active, $S=^1/_2$ FeMoco(CN)^{superox} level.

We find that electrogenerated FeMoco(CN)^{superox} is capable of restoring ethyne reduction activity to an NifB$^-$ mutant nitrogenase FeMo protein lacking the FeMoco centre,[18] to a level 80±5% of that observed on reconstitution with FeMoco^{semired}. Importantly, this shows that forming the cyanide-ligated superoxidised level does not destroy the core structure of the native cofactor, consistent with the reversibility of the redox processes and cyanide-binding chemistry.

We have argued previously that processes **I** and **II** are associated with electron transfer to the delocalised iron–sulfur core of FeMoco to generate the semireduced and reduced states, respectively, whereas process **III** is centred on the Mo atom, and accesses a superreduced level of the cluster.[6] The dramatic shift in the redox potential of the oxidised/semireduced couple on cyanation to more negative potentials ($\Delta E=360$ mV) is consistent with the binding of a σ-donating anionic ligand to the delocalised iron core of FeMoco^{ox}. This increases the electron density on the core, thereby raising the energy of the lowest unoccupied molecular orbital (LUMO). This same charge effect is also responsible for allowing access to the new superoxidised/oxidised couple.

Notably, the difference in potential between the superoxidised/oxidised and oxidised/semireduced couples of the cyanide-modified form of the cofactor is 460 mV, a magnitude consistent with significant delocalisation over the cluster core.[6,19] Thus, cyanide can be viewed as perturbing the redox manifold of the iron–sulfur core, as illustrated by Scheme 1. The semireduced/reduced couple is apparently shifted outside the accessible potential range on cyanide binding.

Figure 4a shows thin film IR differential absorption spectra recorded for a solution of native FeMoco^{ox} in the presence of excess $[Et_4N]CN$, following a potential step from



Scheme 1. Redox potentials for the levels of FeMoco(SPh) and the shift in these levels on binding cyanide and co-binding CO. The position of CO/CN ligands on particular central Fe atoms is arbitrary.

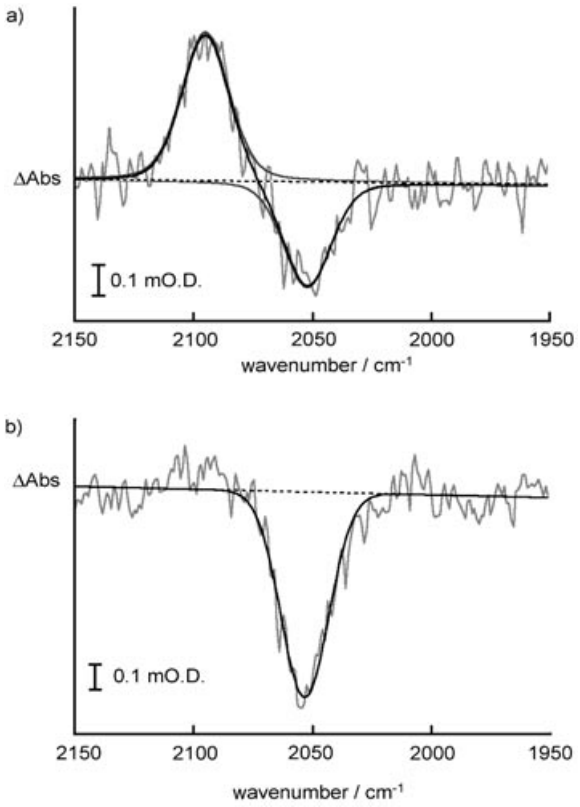


Figure 4. Differential FTIR absorption spectra recorded for a solution of FeMoco in the presence of excess cyanide following a) oxidation at approximately −0.3 V and b) reduction at about −1.1 V. The background trace for both spectra shown was recorded with the electrode held at an initial potential of approximately −0.6 V. Spectra were calculated from an average of 100 scans recorded over about 100 seconds following application of the final potential. Raw data (pale grey line), fitted peaks (dark grey line), baseline (dotted line) and the resulting fitted trace (black line) are overlaid.

−0.6 to −0.3 V. A single depletion band at 2053 cm^{-1} and a single growth band at 2095 cm^{-1} are observed. Ligation of free cyanide (ν(CN) = 2070 cm^{-1}, NMF) results in a marked increase in intensity of the ν(CN) stretch, with the wavenumber highly sensitive to the oxidation state of the metal centre (generally 2200 to 2000 cm^{-1}).[20] Thus, the depletion and growth features are attributed to the conversion of the parent cyano species FeMoco(CN)^{ox} to FeMoco(CN)^{superox}. Consistent with the cyclic voltammetry, the spectroscopic changes are fully reversible on re-reduction (data not shown). The shift in ν(CN) to higher wavenumber following oxidation is concordant with strengthening of the CN bond, resulting from enhanced σ donation to the metal from the ligand 5σ molecular orbitals (weakly antibonding with respect to the CN bond) and diminished involvement of the ligand 2pπ* molecular orbital in π back-bonding with the metal.[20]

The magnitude of the band shift ($\Delta\nu$(CN) = 42 cm^{-1}) between the two redox states relative to that of the mononuclear ferri-/ferrocyanide couple ($\Delta\nu$(CN) = 37 cm^{-1}, H_2O),[21] clearly indicates that the cyanide ligand is bound to a metal site on the cofactor that is strongly coupled to the redox change. Since the analysis of the electrochemistry and EPR spectra discussed earlier indicates that the electron transfer associated with the FeMoco(CN)$^{superox/ox}$ redox couple is delocalised over the iron core, we conclude that it is cyanide bound to iron site(s) that is responsible for the 2095 (superoxidised) and 2053 cm^{-1} (oxidised) bands. It is most likely that CN^- ligates central iron sites, since processes **O** and **R** are observed following addition of CN^- in the voltammetry of both FeMoco and FeMoco(SPh), and EPR titration data reported by Richards et al. have demonstrated that CN^- does not appear to compete with SPh^- for binding at Fe_{term}.[13] We have argued that the molybdenum atom is essentially electronically insulated from the core iron–sulfur assembly,[6] and consequently we would not necessarily expect to detect a different spectral response at the superoxidised, oxidised and semireduced levels associated with cyanide bound to Mo.

Reduction of FeMoco(CN)^{ox} (potential step from −0.6 V to −1.1 V) results in a single ν(CN) depletion band at 2053 cm^{-1} (Figure 4b). Since reduction generally leads to an increase in intensity of ν(CN), the absence of a well-defined growth band at lower wavenumber confirms that cyanide is lost from FeMoco^{semired} in the time frame of the spectroelectrochemical data collection (about 100 s). This is fully consistent with the partial reversibility of the voltammetric response at process **R** (Figure 2, inset). Unambiguously, raising the electron density on the cluster core labilises cyanide.

Each single sharp ν(CN) band associated with the superoxidised and oxidised redox levels of FeMoco(CN) is well fitted by a single Gaussian/Lorentzian curve (Figure 4). This is consistent with a single cyanide bound to the iron core at each redox level. Moreover, if two cyanide ligands were bound to electronically similar iron sites, it would be anticipated that the labilisation of cyanide upon reduction to the semireduced level would proceed in two separate steps, and this would be apparent both in the spectroscopy and the voltammetry.

On the basis of recent ligand displacement kinetic studies, supported by DFT calculations, Henderson and co-workers have concluded that the Mo atom is the site of strongest cyanide binding to the dithionite-reduced cofactor (FeMoco$^{semi-red}$).[10] This is consistent with labilisation of the ligand at iron core sites of the cofactor at this redox level. Detailed analysis of EXAFS data has also provided evidence for the molybdenum as a site of CN^- binding on the dithionite-reduced cofactor.[12] We do not exclude cyanide also binding to the molybdenum; however, the localised redox chemistry of this centre involves the reduced and superreduced levels, which we have not accessed in spectroelectrochemical measurements. No change in the potential of the Mo-localised FeMoco$^{red/superred}$ couple (process **III**) was observed on addition of cyanide (Figure 2, inset); thus if CN^- does interact with Mo under these conditions, it results in no perceptible change in the electron density at the Mo centre, perhaps because another anionic donor ligand, such as a carboxylate arm of homocitrate or a deprotonated solvent molecule, is displaced on CN^- binding. Conradson et al. attributed a loss of the ^{19}F NMR signal associated with dithionite-reduced FeMoco(p-$CF_3C_6H_4S$), following addition of approximately 5 molar equivalents of CN^-, to a broadening of the ^{19}F reso-

nance resulting from an increase in the electronic relaxation time upon CN^- ligation.[11] Given that labilisation of CN^- on accessing FeMoco^{semired} is clear from our electrochemical and spectroelectrochemical data, it is likely that Conradson et al. detected an effect from CN^- bound at Mo. However, it is also possible that the electron-withdrawing nature of the fluorinated thiolate ligand at the terminal Fe site is sufficient to stabilise CN^- on the iron–sulfur core of the dithionite-reduced cofactor.

We have shown in earlier work that carbon monoxide binds to FeMoco and FeMoco(PhS) only on accessing the reduced and superreduced levels, and herein that cyanide binds to the cofactor at the superoxidised and oxidised levels, but only weakly to the Fe core at the semireduced level. Evidence for the cooperative binding of cyanide and carbon monoxide at the oxidised and semireduced levels of the cofactor is now presented.

The voltammetric response obtained when a solution of FeMoco saturated with carbon monoxide at 1 bar is titrated with cyanide is shown in Figure 5. The FeMoco$^{ox/semired}$ couple (process **I**) is unaffected by carbon monoxide

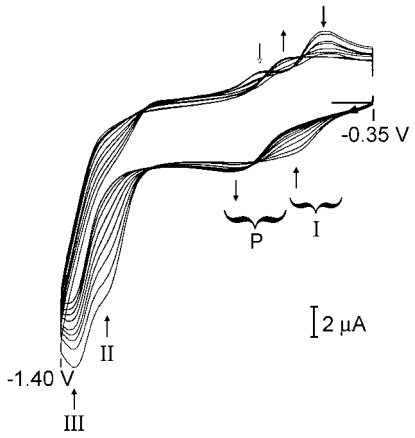


Figure 5. Cyclic voltammograms recorded at 50 mV s^{-1} for an NMF solution of FeMoco saturated with carbon monoxide at 1 bar during titration with $[Et_4N]CN$ (0 to 8 molar equivalents). Vertical arrows indicate the direction of change of the current response with increasing cyanide concentration; the grey arrow highlights a process associated with CO alone bound to the cofactor, as discussed in reference [6].

alone;[6,22] however, as cyanide is introduced, process **I** is replaced by a new, reversible couple at more negative potential (process **P**, −0.71 V). The semireduced/reduced couple (process **II**), well resolved in the presence of carbon monoxide,[6] is steadily lost as the concentration of cyanide is increased (Figure 5). The reoxidation process indicated by a grey arrow in Figure 5 is associated with CO alone bound to the cofactor,[6] and is also lost as the concentration of CN^- is increased.

Infrared spectroelectrochemical data, obtained for a CO-saturated solution of FeMoco in the presence of excess cyanide, exhibit growth and depletion features in both the ν(CN) and ν(CO) regions when the electrode potential is stepped over a range spanning process **P**, −0.5 to −0.9 V, (Figure 6a). Assignment of the spectral features has been confirmed by ^{13}CO labelling (Figure 6b). The data confirm that both cyanide and carbon monoxide are bound to the cofactor at the oxidised and semireduced levels. In the

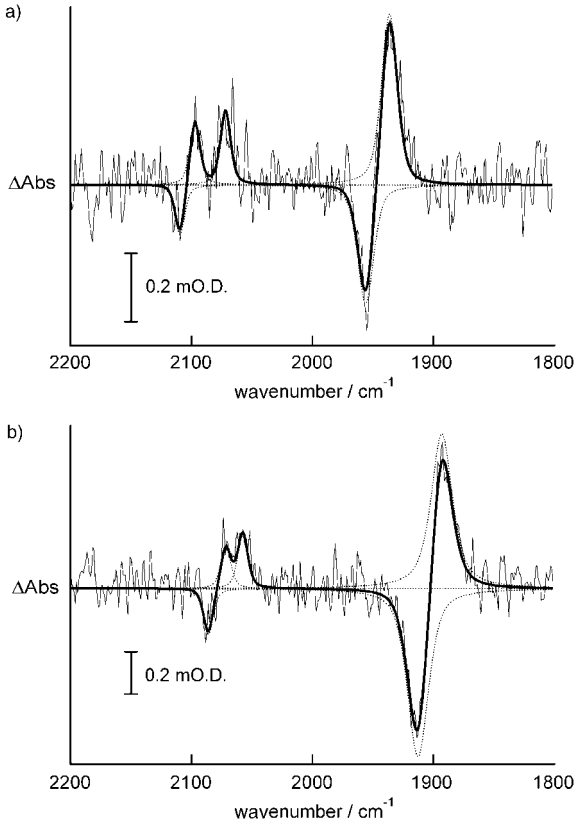


Figure 6. Differential absorption FTIR spectra (thin black line) in the ν(CN) and ν(CO) regions for a solution of FeMoco reacted with a large excess of cyanide under a carbon monoxide atmosphere at 2.7 bar, following reduction at −0.9 V from an initial potential of −0.5 V: a) natural abundance CN^- and CO; b) natural abundance CN^- with ^{13}CO. Fitted peaks (dotted) give rise to the fitted spectrum (heavy black line).

ν(CO) region, a single depletion band is observed at 1957 cm^{-1} and a single growth band at 1937 cm^{-1} (Figure 6a); these are attributed to CO bound at the oxidised and semireduced levels, respectively. Reoxidation leads to a full reversal of the spectral changes, confirming that the system is reversible.[23] These results are in stark contrast to those obtained for reduction of FeMoco^{ox} to the semireduced level in the presence of CO alone; under these conditions, no ν(CO) features are observed.[6] The wavenumber of the ν(CO) band for the semireduced cofactor in the presence of CO and CN^- (1937 cm^{-1}) is substantially higher than that observed for CO alone bound at the iron core of the cofactor at the reduced level in the absence of cyanide, which occurs at 1880 cm^{-1}.[6]

The magnitude of the ν(CO) wavenumber shift with the one-electron redox change (oxidised/semireduced, 20 cm^{-1}) is small compared with that typically observed for one-electron redox chemistry of carbonyl groups at a mononuclear centre, but consistent with charge delocalisation across a polymetallic assembly.[24]

As expected, the $\nu(^{12}CO)$ bands are shifted on isotopic ^{13}CO substitution by 44 cm^{-1} (Figure 6b). Although less intense and less well defined, it is apparent that the ν(CN) bands are also shifted (by about 20 cm^{-1}) upon ^{13}CO substitution. This indicates strong coupling between the CO and CN stretching modes and is consistent with co-binding of CO and CN^- at the oxidised and semireduced levels. The mixing of the CO and CN stretching coordinates is extremely small for Fe(CN)(CO) compounds that have near-orthogonal alignment of the CO and CN groups, as in the H cluster of the Fe-only hydrogenase and model systems.[25,26] We conclude, therefore, that the strong coupling observed on FeMoco must arise from CO and CN^- groups co-aligned on the Fe_6S_9N core, and are not bound to a single Fe or Mo atom.

For spectra recorded under either natural abundance CO or ^{13}CO, the ν(CN) band profiles can most simply be fitted to a single depletion band and two growth bands (Figure 6). At the semireduced level, the ν(CN) bands can be explained by two cyanide ligands vibrationally coupled to a single CO ligand. Although only a single ν(CN) depletion band is observed at the oxidised level, a second band may be masked by growth bands. Alternatively, structural rearrangement or charge localisation may render two cyanide ligands inequivalent at the semireduced level.

We have noted previously that electrocatalytic proton reduction by isolated FeMoco at the oxidised/semireduced level in the presence of the acid pentafluorothiophenolate is suppressed by cyanide.[4] Our electrochemical data, obtained with isolated FeMoco, also allow us to explain data obtained with the functioning enzyme. Cyanide inhibits total electron flux through the enzyme. If the potential of FeMoco in the enzyme observed upon isolation is lowered on binding cyanide, then the transfer of electrons to FeMoco from the P clusters would be less effective and electron flux would be diminished as observed. Furthermore, the effect of carbon monoxide in reversing the cyanide-induced lowering of the isolated FeMoco redox potential, is also paralleled in enzyme studies where the cyanide inhibition of electron flux is countered by low levels of carbon monoxide. Our studies with the isolated FeMoco show co-binding of carbon monoxide and cyanide, and so it is not necessary to suggest that cyanide and carbon monoxide compete for the same site in the enzyme, although this cannot be ruled out.

Summary

A combination of electrochemical, EPR and FTIR spectroelectrochemical data on the isolated nitrogenase cofactor has demonstrated that cyanide can coordinate to central iron sites of oxidised FeMoco, dramatically increasing the electron density on the iron–sulfur core such that the oxidised/semireduced couple is shifted to a significantly more negative potential and a new $S=^1/_2$, superoxidised level becomes accessible. Cyanide is labile at the semireduced level of the cofactor. Co-binding of CO and CN^- at isolated FeMoco has been demonstrated for the first time and FTIR ^{13}C isotopic labelling indicates vibrational coupling of CO and CN^- ligands. This tends to exclude *cis* binding of CO and CN^- at a single metal site(Mo or Fe), but is consistent with co-aligned CO and CN^- at iron on the rigid central Fe_6S_9N core of the cofactor, at both the oxidised and semireduced levels. The shift in the oxidised/semireduced couple in a positive direction on CO/CN^- binding, with respect to CN^- binding alone, could account for the CO relief of cyanide inhibition of proton reduction by nitrogenase. Growth of a strain of *Klebsiella oxytoca* on cyanide under N-limiting conditions has recently been described and has been attributed to the nitrogenase in this organism reducing CN^- to ammonia and methane.[27] This perhaps intimates a vestigial archaic chemistry of the modern, evolved cofactor.

Experimental

The FeMoco cluster was extracted from the nitrogenase FeMo protein of wild-type *Klebsiella pneumoniae*, strain M5a1, into NMF containing water (approximately 2%), phosphate buffer and sodium dithionite, using minor modifications of the methods described previously.[28] The cofactor was concentrated in vacuo to between 0.6 and 1 mM (based on Mo determination, Southern Analytical, Brighton, UK). The integrity of the isolated FeMoco was confirmed by acetylene reduction activity in an enzyme reconstitution assay (activity >275 units mol^{-1} atom Mo), by EPR spectroscopy and by cyclic voltammetry. The $FeMoco^{ox}$ state was obtained by storing the fresh, concentrated extract under dinitrogen for 24–48 h, as described by Schultz and co-workers.[15]

The EPR measurements were performed at 10 K, 100 mW using a Bruker ER 200 D-SRC instrument.

Electrochemical measurements were performed in a glove box maintained at <1 ppm O_2 (Alvic Scientific Containment Systems, UK), using a potentiostat-type DT 2101 and waveform generator PPRI (Hi-Tek Instruments, UK). The electrochemical cell consisted of an open glass vial, incorporating a vitreous carbon working electrode (area 0.070 cm^2), a platinum auxiliary electrode and a saturated calomel reference electrode (SCE). The vitreous carbon electrode was polished with a 0.015 μm alumina slurry in ethanol prior to use.

The FTIR spectroelectrochemical measurements were performed using a BioRad FTS 175C instrument. The absorption/reflection spectroelectrochemical cell incorporating a vitreous carbon working electrode has been described previously.[29] Fitting of peaks to mixed Gaussian/Lorentzian functions was performed using Grams-based routines implemented within WinIR (BioRad) software. Spectra were baseline corrected prior to fitting.

Carbon monoxide and ^{13}CO (99.2 atom% ^{13}CO) were obtained from BOC (UK) and Trace Sciences International (Ontario, Canada), respectively, and tetraethylammonium cyanide was purchased from Aldrich.

Acknowledgement

We thank the BBSRC and the ARC for funding this work; the Bennett Fund for providing a travel scholarship to K.A.V.; the ARC International Exchange Scheme (IREX) for providing travel funds to K.A.V., S.P.B. and C.J.P.; and the John Innes Foundation and the Leverhulme Trust for Emeritus Fellowships to BES.

[1] R. P. Happe, W. Roseboom, A. J. Pierik, S. P. J. Albracht, *Nature* **1997**, *385*, 126.
[2] Y. Nicolet, C. Piras, P. Legrand, C. E. Hatchikian, J. C. Fontecilla-Camps, *Structure* **1999**, *7*, 13–23.
[3] B. K. Burgess, D. L. Lowe, *Chem. Rev.* **1996**, *96*, 2983–3011.

[4] T. L. Le Gall, S. K. Ibrahim, C. A. Gormal, B. E. Smith, C. J. Pickett, *Chem. Commun.* **1999**, 773–774.
[5] O. Einsle, F. A. Tezcan, S. L. A. Andrade, B. Schmid, M. Yoshida, J. B. Howard, D. C. Rees, *Science* **2002**, *297*, 1696–1700.
[6] C. J. Pickett, K. A. Vincent, S. K. Ibrahim, C. A. Gormal, B. E. Smith, S. P. Best, *Chem. Eur. J.* **2003**, *9*, 76–87.
[7] J. Li, B. K. Burgess, J. L. Corbin, *Biochemistry* **1982**, *21*, 4393–4402.
[8] R. Fani, R. Gallo, P. Liò, *J. Mol. Evolution* **2000**, *51*, 1–11.
[9] S. K. Ibrahim, K. Vincent, C. A. Gormal, B. E. Smith, S. P. Best, C. J. Pickett, *Chem. Commun.* **1999**, 1019–1020.
[10] Z. Cui, A. Dunford, M. C. Durrant, R. A. Henderson, B. E. Smith, *Inorg. Chem.* **2003**, *42*, 6252–6264.
[11] S. D. Conradson, B. K. Burgess, S. A. Vaughn, A. L. Roe, B. Hedman, K. O. Hodgson, R. H. Holm, *J. Biol. Chem.* **1989**, *264*, 15967–15974.
[12] H. I. Liu, A. Filipponi, N. Gavini, B. K. Burgess, B. Hedman, A. D. Cicco, C. R. Natoli, K. O. Hodgson, *J. Am. Chem. Soc.* **1994**, *116*, 2418–2423.
[13] A. J. Richards, D. L. Lowe, R. L. Richards, A. J. Thomson, B. E. Smith, *Biochem. J.* **1994**, *297*, 373–378.
[14] Redox levels of the cofactor successively more reduced than FeMoco^{ox} are labelled semireduced (FeMoco^{semired}), reduced (FeMoco^{red}), and superreduced (FeMoco^{superred}), respectively; a more oxidised level is labelled superoxidised (FeMoco^{superox}).
[15] F. A. Schultz, S. F. Gheller, B. K. Burgess, S. Lough, W. E. Newton, *J. Am. Chem. Soc.* **1985**, *107*, 5364–5368.
[16] All potentials are directly referenced to the saturated calomel reference electrode (SCE) in the NMF electrolyte; $E(\mathrm{NHE}) = E(\mathrm{SCE}) + 0.241$ V (298 K).
[17] J.-M. Mouesca, B. Lamotte, *Coord. Chem. Rev.* **1998**, *178–180*, 1573–1614.
[18] T. R. Hawkes, B. E. Smith, *Biochem. J.* **1984**, *223*, 783–792.
[19] C. J. Pickett, *J. Chem. Soc., Chem. Commun.* **1985**, *6*, 323–326.
[20] K. Nakamoto, *Infrared and Raman Spectra of Inorganic and Coordination Compounds, Part B*, 5th ed., Wiley-Interscience, New York, **1997**, pp. 105–115.
[21] K. R. Dunbar, R. A. Heintz, *Prog. Inorg. Chem.* **1997**, *45*, 283–391.
[22] In contrast to the sharp response observed for the FeMoco-(SPh)$^{ox/semired}$ couple, process **I** is broadened in the absence of thiophenolate, probably due to redox-linked isomerism at the terminal Fe site as discussed previously, see reference [15].
[23] Preliminary spectroelectrochemical experiments were conducted in which the potential was stepped out to more negative values so as to access further reduced levels of the cofactor in the presence of CO and CN^-. Under these conditions, the spectra resembled those obtained for the cofactor under CO alone (see reference [6]). This suggests that cyanide may dissociate from the cofactor at these redox levels.
[24] J. D. Roth, G. J. Lewis, L. K. Safford, X. Jiang, L. F. Dahl, M. J. Weaver, *J. Am. Chem. Soc.* **1992**, *114*, 6159–6169.
[25] A. J. Pierik, W. Roseboom, R. P. Happe, K. A. Bagley, S. P. J. Albracht, *J. Biol. Chem.* **1999**, *274*, 3331–3337.
[26] A. Le Cloirec, S. P. Best, S. Borg, S. C. Davies, D. J. Evans, D. L. Hughes, C. J. Pickett, *Chem. Commun.* **1999**, 2285–2286.
[27] C. M. Kao, J. K. Liu, H. R. Lou, C. S. Lin, S. C. Chen, *Chemosphere* **2003**, *50*, 1055–1061.
[28] P. A. McLean, D. A. Wink, S. K. Chapman, A. B. Hickman, D. M. McKillop, W. H. Orme-Johnson, *Biochemistry* **1989**, *28*, 9402–9406.
[29] S. J. Borg, S. P. Best, *J. Electroanal. Chem.* **2002**, *535*, 57–64.

Received: April 20, 2004
Published online: August 17, 2004

