The isolated iron–molybdenum cofactor of nitrogenase binds carbon monoxide upon electrochemically accessing reduced states

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The first spectroscopic evidence for the binding of a small gaseous molecule to the isolated iron molybdenum cofactor of nitrogenase (FeMoco) is presented: FTIR spectroelectrochemistry in a thin-layer cell shows that reduced FeMoco binds carbon monoxide and gives rise to ν(CO) stretches that are close to those observed during turnover of the enzyme.

The cofactor extract[1–3] in NMF was in the oxidised EPR-silent form, FeMoco^{ox}.† Cyclic voltammetry of FeMoco^{ox} at a vitreous carbon electrode is shown in Fig. 1. The primary reduction process [A in Fig. 1(a)] encompasses the one-electron reduction of the oxidised cofactor to the EPR-active (S = 3/2) FeMoco$^{semi\text{-}red}$ state, a process which involves reversible interconversion of redox isomers, as delineated earlier by Schultz *et al.*[4] The reduction of the semi-reduced state is observed as a poorly resolved irreversible process under argon or molecular nitrogen [B in Fig. 1(a)]. Whereas the primary process A is unperturbed by carbon monoxide, interaction with CO is evident by the resolution of process B into two successive reduction steps [C and D in Fig. 1(b)] and by the detection of two oxidisable species [E and F in Fig. 1(b)]. Purging the solution with argon or dinitrogen fully restores the response to that observed in the absence of CO. Importantly, an enzyme reconstitution assay[5] showed the cofactor to be fully active after exposure to CO for one hour.

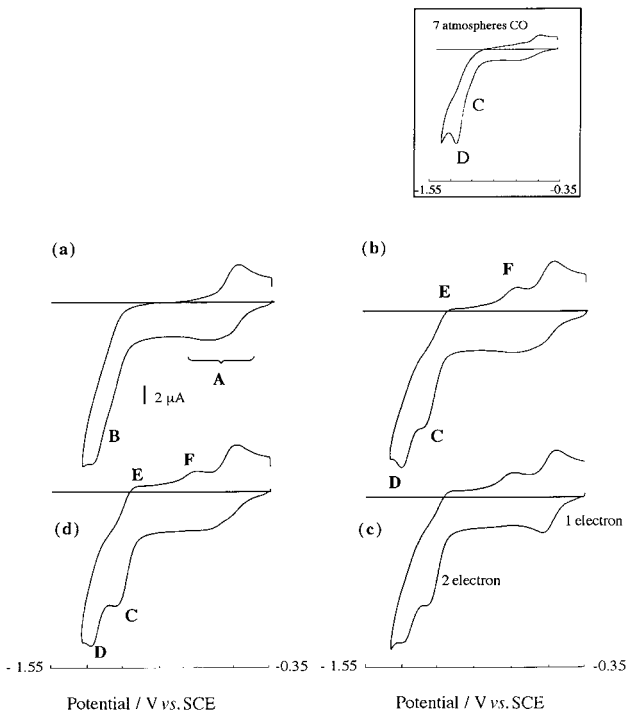

Fig. 1 (a) Cyclic voltammogram of FeMoco^{ox} (*ca.* 1.8 mM) in NMF recorded at a vitreous carbon electrode of area 0.071 cm^2 at 292 K under a dinitrogen atmosphere in a glove box operating at < 1 ppm O_2, the potential scan rate = 50 mV s^{-1}; (b) conditions as for (a) but saturated with CO at 1 atm; (c) conditions as for (b) after the addition of 1 equiv. of PhSH, the isomeric system A collapses to a single reversible one-electron process corresponding to thiolate ligation at the terminal Fe atom of the cofactor; (d) conditions as for (b) in the presence of 90 mM imidazole. Inset: cyclic voltammogram of FeMoco^{ox} recorded under CO at 720 KPa showing the increase in the height of peak D, conditions otherwise as for (b) except [FeMoco^{ox}] *ca.* 1 mM.

Variable scan-rate studies show that the intermediates detected at E and F are generated by the CO-dependent two-electron reduction process occurring at C. Process D involves further reduction and binding of CO. With increasing CO pressure, peak D increases in intensity and is shifted to more positive potentials; this is indicative of a rate-limiting interaction with CO, Fig. 1 (inset). Carbon monoxide is a powerful π-*acid* ligand, thus binding to the cofactor not unexpectedly promotes sequential two-electron transfer steps (processes C and D) *cf.* proton-coupled redox chemistry.[6]

Despite the nominal coordinatively unsaturated nature of the trigonal iron atoms of the Fe_7S_9Mo cluster in the protein and (presumably) in the isolated cofactor, neither the resting-state MoFe-protein nor the corresponding FeMoco$^{semi\text{-}red}$ state of the cofactor, interact measurably with CO. Turnover is necessary to observe MoFe-protein–CO interactions; accessing the FeMoco^{red} state is necessary to observe isolated cofactor interactions with CO, at least at pressures below 2016 kPa.

Since the cyclic voltammetry suggests that at least four electrons can be added overall to the FeMoco$^{semi\text{-}red}$ state under CO, it would not be surprising if redox chemistry associated with accessing low valent Fe and/or Mo states is involved.[7]

Direct spectroscopic evidence for the binding of carbon monoxide to reduced states of FeMoco is revealed by thin-layer FTIR spectroelectrochemistry[8] under moderate pressures (720 kPa) of CO at a highly polished vitreous carbon working electrode (2.6 mm diameter disc). Potential-dependent FTIR spectra obtained by reflectance through the thin solution layer (*ca.* 10–20 μm) are shown in Fig. 2. Two major bands develop at 1883 and 1922 cm^{-1} which we assign to terminally bound CO at reduced FeMoco states. Neither band appears under argon or dinitrogen, nor in a solution of the cofactor irreversibly damaged by exposure to air.

The band at 1883 cm^{-1} is the first to appear at high potential [compare Fig. 2(a)–(c)] and is therefore most likely associated with process C, that at 1922 cm^{-1} becomes dominant at lower potentials and is probably associated with process D [Fig. 1(b)]. Re-oxidation at E_{appl} −0.85 V *vs.* SCE selectively depletes the 1883 cm^{-1} band and gives rise to a strong new absorption at 1965 cm^{-1} and a weak band at 1999 cm^{-1} (data not shown). Re-oxidation at E_{appl} −0.45 V *vs.* SCE depletes both infrared bands. It is probable that these two oxidations are associated with processes E and F, respectively, Fig. 1(b).

Coordination of thiophenol at the terminal tetrahedral Fe atom and imidazole (Im) at the Mo atom provides a ligation environment for FeMoco akin to that of the cofactor in the

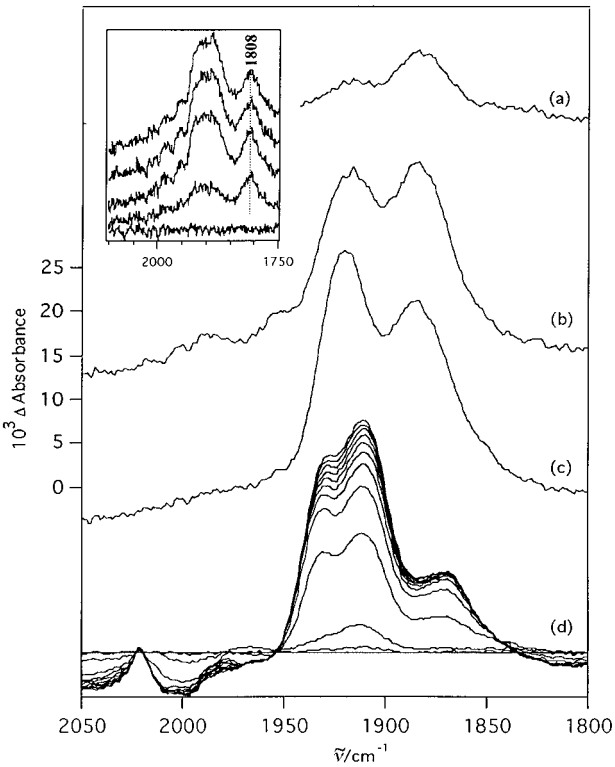

Fig. 2 Thin-layer FTIR spectroelectrochemistry of FeMoco at a highly polished vitreous carbon disc of area 0.053 cm^2 at ambient temperature. (a) Accumulated spectrum after electrolysis under CO at 720 KPa for 70 s at E_{appl} −1.05 V *vs.* SCE showing growth of bands centred on 1883 and 1922 cm^{-1}; (b) as for (a) but at E_{appl} −1.25 V *vs.* SCE; (c) as for (a) but at E_{appl} −1.45 V *vs.* SCE; (d) as for (c) showing the development of bands centred at 1870, 1910 and 1929 cm^{-1} when reduction is performed in the presence of thiophenol (*ca.* 100 mM) and imidazole (*ca.* 50 mM). Inset: development of spectra at low (pre-equibrium) concentrations of CO showing development of a band at 1808 cm^{-1}, the spectra were recorded at 12.4 s intervals.

enzyme.[1–3,9] Whereas FTIR spectra obtained in the presence of thiophenol are similar to those observed for the unmodified cofactor, the addition of imidazole has a substantial effect. This is shown by Fig. 2(d), the 1922 cm^{-1} band is split into two new bands which appear at 1910 and 1929 cm^{-1} and which develop at the same rate. This suggests that at least two CO molecules can be bound at the same cofactor molecule. Additionally, the band at 1883 cm^{-1} is shifted to 1870 cm^{-1}.

Cyclic voltammetry shows that ligation of thiophenolate collapses the redox isomerism of the FeMoco$^{ox/semi\text{-}red}$ system to that of a single reversible couple,[4] but does not otherwise perturb the voltammetry under CO [Fig. 1(c)], thus the terminal tetrahedral Fe-atom of the cofactor is unlikely to be a site at which CO binds. Imidazole does not change the form of the voltammetry under CO even at [Im] = 90 mM [Fig. 1(d)]. Evidently imidazole and CO do not compete for the same site at Mo, as is concordant with the spectroelectrochemistry.

George *et al.*[10] have recently reported that turnover of nitrogenase under CO at low partial pressure leads to the growth of a band at 1906 cm^{-1} which is replaced by an intense absorption near 1936 cm^{-1} at higher partial pressures of CO; weaker absorptions near 1958 and 1880 cm^{-1} also appear. The major bands we observe, particularly those obtained in the presence of imidazole and thiophenol, clearly fall in the spectral domain of CO interactions with the enzyme.

Hoffman and Hales[11] have proposed a model for CO interaction with the {Fe$_7$S$_9$Mo} cluster which is based on detailed isotopic EPR and ENDOR studies of the enzyme during turnover. At low [CO] they suggest the binding of CO in a bridging or semi-bridging fashion between two core Fe atoms; at high [CO] a second molecule then binds to one of these Fe atoms thereby opening the bridge and giving a species with two terminally bound CO ligands, one on each of the neighbouring Fe sites. *A priori* their data do not exclude CO binding at molybdenum nor the generation of EPR-active states produced by two- or four-electron reduction of the resting state MoFe-protein, *cf.* process C and D.

Whilst the major bands we observe for the cofactor are undoubtedly associated with terminally bound CO ligands,[12] at low [CO] a weak band at 1808 cm^{-1} precedes the growth of the 1883 and 1922 cm^{-1} absorptions, Fig. 2 (inset). This may arise from a bridging carbonyl intermediate, related to that proposed by Hoffman and Hales,[11] however a terminal CO stretch cannot be excluded.

In conclusion, direct studies of the isolated cofactor have certain advantages over studies on the whole enzyme system, notably the possibility of selectively accessing redox states; the exclusion of complications which might arise from the presence of the other metallo-sites within the MoFe- or Fe-proteins; and the opportunity to widely and systematically control both the co-ligand and the outer-sphere environment. The studies described herein take some first steps towards obtaining spectroscopic information on interactions of isolated FeMoco at redox levels not hitherto addressed, and which are complementary to studies of the whole enzyme system under turnover conditions.

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Notes and references

† *Ca.* 1.8 mM FeMoco in NMF containing Na_2HPO_4 phosphate buffer (*ca.* 100 mM) and water (*ca.* 5% v/v). The NMF spectroscopic window is 2300–1720 cm^{-1}.

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