A new approach to identifying substrate binding sites on isolated FeMo-cofactor of nitrogenase

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A kinetic method is described which is capable of detecting the binding of molecules and ions such as CN^-, N_3^-, Bu^tNC, imidazole and H^+ to the isolated FeMo-cofactor of nitrogenase, and indicates where on the cofactor these species bind.

The Mo-based nitrogenase converts dinitrogen to ammonia by a sequence of electron- and proton-transfer reactions, and a variety of other small, unsaturated molecules are similarly transformed. There is strong evidence that these substrates are converted into products at a $MoFe_7S$(homocitrate) cluster: the FeMo-cofactor[1,2] (Fig. 2) which can be extracted into *N*-methylformamide (NMF), free from the polypeptide. Fundamental to the understanding of nitrogenase is identification of where these substrates bind on this cluster.[3] Herein, we report the novel approach of using kinetics to detect the binding of a variety of molecules (including some of the substrates of nitrogenase) on extracted cofactor. This approach also indicates where on the cluster this binding occurs.

The principle reaction used in our approach is a simple substitution reaction. Extracted cofactor[4] in the $S = 3/2$ state reacts with one PhS^-/FeMo-co.† It is likely that this reaction occurs at tetrahedral Fe and involves displacement of NMF by PhS^-. When studied on a stopped-flow spectrophotometer, this substitution is associated with an exponential absorbance increase (λ = 400–500 nm), and the rate of the reaction is independent of $[PhS^-]$, with $k_1^{NMF} = 50 \pm 5\ s^{-1}$. These observations are consistent with a mechanism involving rate-limiting dissociation of NMF.

Binding other molecules (L) anywhere on the cluster will perturb the overall electron distribution and would be expected to affect the lability of the Fe–NMF bond. The magnitude of this labilisation gives information about where L binds relative to the site of PhS^- substitution: a large perturbation would be expected from binding close to (or at) the tetrahedral Fe, whereas a small perturbation is expected from binding close to (or at) the remote Mo.[4]

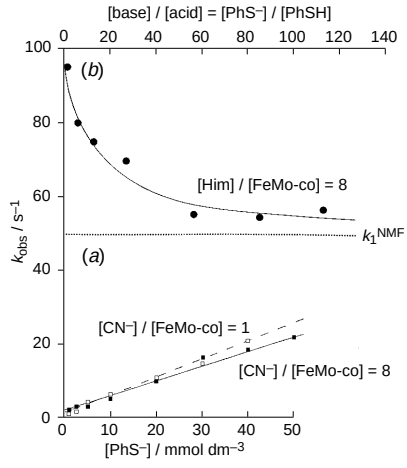

Fig. 1 (*a*) Effect of $[PhS^-]$ on the rate of the reaction with CN^- and FeMo-cofactor and (*b*) effect of $[PhS^-]_f/[PhSH]_f[PhSH]_f$ ($[PhS^-]_0$ = 0–50 mmol dm^{-3}) on the rate of the reaction of PhS^- with imidazole and FeMo-cofactor. The value of k_1^{NMF} is shown as a dotted line at $k_{obs} = 50\ s^{-1}$.

The addition of L = C_2H_2 or CO to the cofactor has no effect on the rate of the reaction with PhS^-, and it must be concluded that these molecules do not bind to the cofactor in the $S = 3/2$ state. However, for all other species studied (L = N_3^-, Bu^tNC, CN^-, imidazole or H^+) perturbations to the rate are observed.

Addition of CN^- to the cofactor ($[CN^-]/[FeMo\text{-}co] = 1.0$) affects the reaction with PhS^- in two ways (Fig. 1). First, the substitution reaction is slower ($k_1^{NMF}/k_1^{CN} = 62.5$). Secondly, the rate law contains two terms: one exhibits a first-order dependence on $[PhS^-]$ the other is independent of PhS^-. These observations are consistent with CN^- being bound to tetrahedral Fe and displacement by PhS^- occurring *via* the associative (k_2^{CN}) and dissociative (k_1^{CN}) pathways shown in Fig. 2.

The addition of excess CN^- ($[CN^-]/[FeMo\text{-}co] \geqslant 2$) leads to further perturbations of the rate: k_1^{CN} is increased by a factor of 1.7, whilst k_2^{CN} is decreased by a factor of 1.2. These small effects are consistent with: (*a*) binding of an additional CN^- remote from the tetrahedral Fe (probably at Mo); (*b*) the presence of this cyanide being transmitted through the cluster. Our interpretation that CN^- binds at both the tetrahedral Fe and the Mo is consistent with EPR studies[5] (which indicate that cofactor can bind more than one CN^-) and EXAFS studies[6] (which indicate that when $[CN^-]/[FeMo\text{-}co] = 4$, cyanide is bound to Mo). Of all the molecules studied here, CN^- is the only one which shows binding to more than one site.

The rate of PhS^- substitution is only slightly perturbed on addition of L = N_3^- or Bu^tNC to cofactor (Table 1) which is consistent with these species binding at the Mo end. N_3^- accelerates and Bu^tNC inhibits the rate of reaction with PhS^-, presumably as a consequence of the relative electron-releasing and -withdrawing properties of these coordinated molecules.

In the protein, Mo is bound to the polypeptide by the imidazole group of His-442. Consistent with this, our kinetic approach indicates that imidazole binds at the Mo end of extracted cofactor since in the presence of this molecule the rate of the PhS^- substitution reaction is only slightly perturbed. Interestingly, at

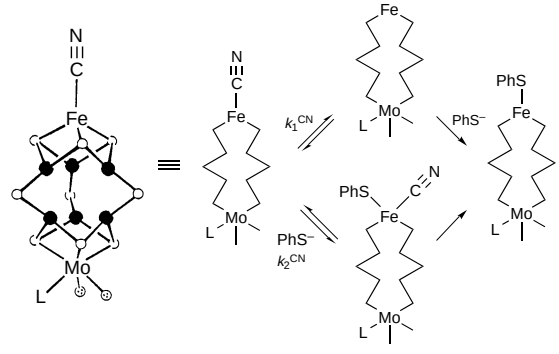

Fig. 2 ● = three-coordinate Fe, ☉ = O (from homocitrate ligand), ○ = S. Proposed pathways for the reaction between FeMo-cofactor (only skeleton shown for clarity) and PhS^- in the presence of CN^-. Rate law: $-d[\text{FeMo-co}]/dt = \{k_1^{CN} + k_2^{CN}[PhS^-]\}[\text{FeMo-co}]$; when $[CN^-]/[FeMo\text{-}co] = 1$, L = NMF, $k_1^{CN} = 0.8\ s^{-1}$, $k_2^{CN} = 5.1 \times 10^2\ dm^3\ mol^{-1}\ s^{-1}$; when $[CN^-]/[FeMo\text{-}co] \geqslant 2$, L = CN, $k_1^{CN} = 1.35\ s^{-1}$, $k_2^{CN} = 4.3 \times 10^2\ dm^3\ mol^{-1}\ s^{-1}$.

Table 1 Indicated substrate binding sites on FeMo-cofactor in $S = 3/2$ state[a]

Substrate (L)	k_1^{NMF}/k_1^{L}	Binding site	Spectroscopic information
CO	1.0	No binding	No change in ^{19}F NMR spectrum of *p*-$CF_3C_6H_4S$–FeMo-co in the presence of CO.[8]
C_2H_2	1.0	No binding	
CN^-			
$[CN^-]/[FeMo\text{-}co] = 1$	62.5	Fe	Large changes in EPR spectrum.[5]
$[CN^-]/[FeMo\text{-}co] \geqslant 2$	37	Mo	EXAFS shows CN^- bound to Mo when $[CN^-]/[Mo] = 4$.[6]
			CN^- affects relaxation time of *p*-$CF_3C_6H_4S$–FeMo-co.[8]
			Stoichiometry experiments using EPR indicates more than one CN^- can bind to FeMo-co.[5]
N_3^-	0.83	Mo	EPR spectrum of FeMo-co[9] sharpens in presence of N_3^-.
Bu^tNC	1.21	Mo	MeNC affects relaxation time of *p*-$CF_3C_6H_4S$–FeMo-co.[8]
Him	0.52	Mo	EPR spectrum of FeMo-co[9] sharpens in the presence of Him.
H^+	0.16	S	Both EPR spectrum and electrochemistry indicated

[a] After several weeks storage of FeMo-co in an anaerobic box the experimental value of k_1^{NMF} gradually increased although activity of FeMo-co is retained. This we attribute to an increase in the concentration of protons in solution due to dithionite decomposition. k_1^{L} is the rate constant when the substrate L is present, and k_1^{NMF} is the rate constant in the absence of L, determined on the same day.

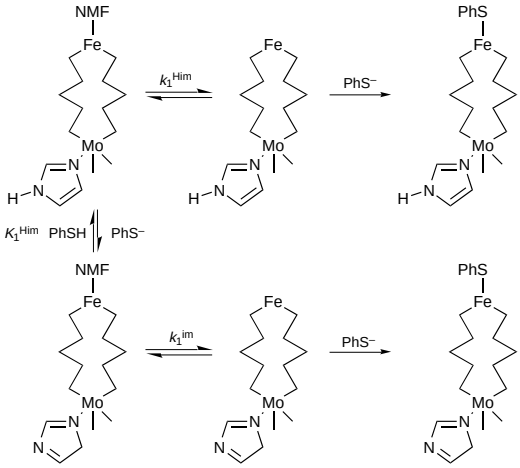

Fig. 3 Proposed pathways for the reaction between FeMo-cofactor (only skeleton shown) and PhS^- in the presence of imidazole. Rate law:

$$\frac{-d[\text{FeMo-co}]}{dt} = \frac{\{k_1^{Him} + k_1^{im}K^{Him}[PhS^-]_f/[PhSH]_f\}[\text{FeMo-co}]}{1 + K_1^{Him}[PhS^-]_f/[PhSH]_f}$$

where $k_1^{Him} = 95\ s^{-1}$, $k_1^{im} = 50\ s^{-1}$ and $K_1^{Him} = 8 \times 10^{-2}$. The subscript 'f' denotes the concentration of 'free' reactant, having made allowance for the protolytic reaction between uncoordinated imidazole and PhS^-: $ImH + PhS^- \rightleftharpoons Im^- + PhSH$. If $[PhS^-]_0 > [Him]_0$ and this reaction goes to completion, (where subscript 0 denotes total concentration of the reagent) $[PhS^-]_f = [PhS^-]_0 - [Him]_0$ and $[PhSH]_f = [Him]_0$.

low $[PhS^-]$ the reaction is accelerated ($k_1^{NMF}/k_1^{Him} = 0.52$), whilst at higher concentrations of PhS^- the acceleration is less marked (Fig. 1). This behaviour is consistent with PhS^- acting not only as a nucleophile to cofactor, but also as a base which can deprotonate imidazole and that the rate of the substitution reaction with PhS^- is sensitive to the state of protonation of the coordinated imidazole. The proposed mechanism is shown in Fig. 3.

Studies on a variety of synthetic Fe/S-based clusters have shown that invariably protons bind to μ-S atoms and increase the substitution reactivity of the cluster.[7] FeMo-cofactor exhibits analogous behaviour with the rate of the PhS^- substitution being faster in the presence of $[NHEt_3]BPh_4$ ($k_1^{NMF}/k_1^{H} = 0.16$).‡ This large acceleration in rate makes it likely that protonation is at a μ-S close to the tetrahedral Fe.[7]

Table 1 shows that the results of our kinetic studies are consistent with earlier studies on binding of molecules at extracted FeMo-cofactor. However, our kinetic approach is more versatile since it circumvents the problem of poor spectroscopic response associated with binding of molecules to cofactor.

This study indicates that various nitrogenase substrates can exhibit a marked selectivity for different sites on cofactor and that there are at least three sites at which substrates can bind. (*i*) The Mo (or a site at this end of the cluster) at which all anionic and neutral substrates bind, (*ii*) tetrahedral Fe at which cyanide prefers to bind, and (*iii*) μ-S where protons bind.

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Footnotes

† FeMo-cofactor from wild type *Klebsiella pneumoniae* M5al was extracted into an NMF solution containing sodium dithionite and sodium phosphate buffer, pH 8, using a modification of the method of P. A. McLean, D. A .Wink, S. K. Chapman, A. B. Hickman, D. M. McKillop and W. H. Orme-Johnson. (*Biochemistry*, 1989, **28**, 9402). In NMF solution, the cluster-protein ligands His-442 and Cys-275 are most likely replaced by solvent molecules. In the kinetic experiments, cofactor was diluted to *ca.* 1×10^{-4} mol dm^{-3} in NMF and reacted with $[NEt_4]SPh$ (0–50 mmol dm^{-3}). In studies with $L = CN^-$, N_3^-, Bu^tNC, CO, C_2H_2 and imidazole, the substrate was added to a solution of FeMo-cofactor (gases added by syringe), such that (for instance) $[L]/[FeMo\text{-}co] = 8$.

‡ The kinetics of this reaction exhibit a dependence on the ratio, $[NHEt_3^+]_f/[NEt_3]_f$:

$$\frac{-d[\text{FeMo-co}]}{dt} = \frac{\{k_1^{H}K^{H}[NHEt_3^+]_f/[NEt_3]_f\}[\text{FeMo-co}]}{1 + K^{H}[NHEt_3^+]_f/NEt_3]_f}$$

The concentration of 'free' reactants, has made allowance for the protolytic equilibrium: $[NHEt_3]^+ + PhS^- \rightleftharpoons NEt_3 + PhSH$.

Since this reaction goes to completion when $[NHEt_3{}^+]_0 > [PhS^-]_0$, $[NHEt^{3+}]_f = [NHEt_3^+]_0 - [PhS^-]_0$ and $[NEt_3]_f = [PhS^-]_0$.

These kinetics describe a mechanism involving rapid protonation of the cofactor ($K^H = 5.6 \times 10^{-2}$), followed by rate-limiting dissociation of the Fe–NMF bond ($k_1^H = 312\ s^{-1}$).

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