NOTIZEN

The Chemical Evolution of a Nitrogenase Model, XXII. Reduction of Acetylene with Catalysts Derived from Molybdate, Homocitric Acid and N-Methylimidazole and a Proposal Concerning the Active Site of Functional Azotobacter Nitrogenases

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Based on model experiments it is suggested that the reduction of substrates by nitrogenase occurs at a mononuclear site containing molybdenum, homocitrate and the imidazole component of his-442 of the apoenzyme, generated from the FeMo-co by a reversible dissociation of the Fe_7S_8 unit from the $MoFe_7S_8$ cluster in a redox-linked process.

Introduction

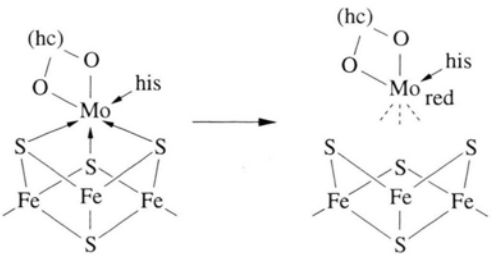
Previous studies indicated [1,2] that the reductions of substrates of bacterial nitrogenases are characteristic of reactions at a mononuclear molybdenum (Mo-) site and that iron-sulfur cluster complexes act as efficient catalysts of electron transfer without otherwise chemically participating in the binding and the reduction of the substrates.

The studies furthermore suggested that Mo in nitrogenase could be bound to either a *cys*-S- or a *his*-imidazole group to the apoprotein [3], and that the Mo atom was in a sterically protected and locally acidic environment [4]. In 1987, *homocitrate* (hc), was found to be an essential component of the FeMo-cofactor [5]. This suggested that this relatively uncommon hydroxycarboxylic acid was essential for the catalytic activity. The 2-OH group and at least one of the carboxylic residues of hc would be expected to coordinate to Mo and possibly render it more readily reducible or catalytically active. In a previous study of the reduction of C_2H_2 by mononuclear Mo(IV) species, glycol, *i.e.* a ligand containing hydroxyl groups for coordination to Mo, was found to increase the reactivity [6]. X-ray crystallographic studies of the FeMo-protein from *Azotobacter vinelandii* [7] and from *Clostridium pasteurianium* [8] have now established that the Mo ion is attached to the *histidine* imidazole nitrogen atom (his$^{\alpha 442}$ Nδ1) of the apoprotein. The second and third coordinating sites of Mo are occupied by oxygen atoms from the hydroxyl group and the secondary carboxyl group of homocitrate (hc). The three remaining coordination sites of molybdenum are made up of three μ-sulfide ions of the Fe_3S_4 unit of the Fe_7S_8 cluster.

Since the X-ray structural analysis represents a resting form of the enzyme, it is difficult to identify the site of substrate binding and reduction. It has been suggested that dinitrogen and other substrates may bind inside the FeMo-cofactor and that Mo, because of its hexacoordinated ligand environment, cannot participate in substrate binding *without either a change in coordination number or a change in liganding groups* [6]. As our previous model studies strongly ruled out iron sites for substrate binding and reduction, we considered a mechanism through which the catalytically active enzymic Mo center, "Mo_{red}(hc)*-his-", would be released by way of a *reversible, redox-linked dissociation* of the enzyme bound FeMo-cofactor-cluster, enz-Fe_7S_8Mo(hc)-his, under the reducing conditions of the functioning enzyme, as indicated in eq. (1):

$$\text{enz-Fe}_7\text{S}_8\text{Mo(hc)-his} + \text{ne}^- \rightarrow \text{enz-Fe}_7\text{S}_8^{-n} + \text{Mo}_{red}\text{(hc)*-his} \quad (1)$$

Support for a process as shown in eq. (1) comes from studies of redox-linked addition/dissociation reactions of ferredoxin clusters [8] and observed reactions of Fe_3S_4 clusters with several different heterometal ions. The generation of Mo_{red}(hc)-his, which is suggested to occur in functional nitrogenase in analogy to these reactions, is shown in to eq. (2):



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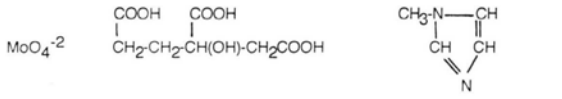
In accord with ref. [8] the Fe_3S_4-unit of the enz-Fe_7S_8 cluster would act as a tridentate ligand, capable of binding the oxidized form of Mo_{red}(hc)-his after completion of each substrate reduction step.

The Mo atom in Mo_{red}(hc)*-his would have 3 coordination sites available, sufficient for the end-on or side-on binding, reduction and protonation of various substrates, while the enz-Fe_7S_8 unit would serve as the electron transfer catalyst. In order to obtain experimental support for this mechanism, the reduction of acetylene (C_2H_2), a widely used alternative substrate of nitrogenase, was investigated with mixtures of MoO_4^{-2}, hc and N-methylimidazole (nmi) as catalyst precursors (see Scheme I); the nitrogen base nmi was chosen as a model for the imidazole group of *his* at the enzymic Mo site.

Scheme I

Model System Components:



Molybdate Homocitric acid (hc) N-methylimidazole (nmi)

Results and Discussion

The reduction of C_2H_2 with aqueous buffered mixtures of Na_2MoO_4, hc and nmi as catalyst precursors occurs upon the addition of reducing agent ($NaBH_4$). The originally colorless solutions turn rapidly brown due to the formation of reduced Mo species which catalyze the reduction of C_2H_2. The most active catalysts resulted from equimolar mixtures of MoO_4^-, nmi and hc, as shown in Fig. 1 and Table I. The reduction takes place without an induction period, see Fig. 2. The catalysts generat-

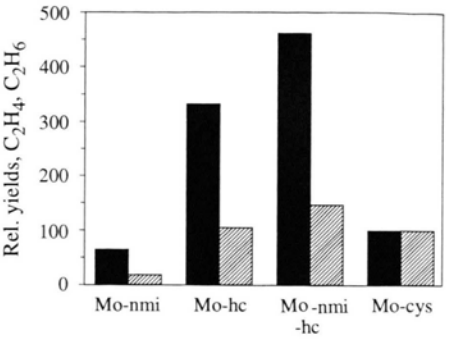


Fig. 1. Yields of C_2H_4 and C_2H_6 from the reduction of C_2H_2 with Mo-nmi-hc systems relative to the yields with molybdocysteine catalysts, with $NaBH_4$ as the reducing agent; $[C_2H_2]_{initial}$ = 223 μmol, $[NaBH_4]_{initial}$ = 7.0 mmol. Reaction solutions contained 14.2 μmol of MoO_4^{-2}, with or without 14.2 μmol of nmi or hc, in a total volume of 2.0 ml of a 1:1 mixture (v/v) of water and 0.05 F borate buffer of pH 9.6. The Mo-cys system contained 14.2 μmol each of MoO_4^{-2} and L(+)cysteine in a total volume of the same solvent; yields were measured after 20 min of reaction at 20 °C. Yields of C_2H_4, black; C_2H_6, shaded; rel. to yields in Mo-cys system.

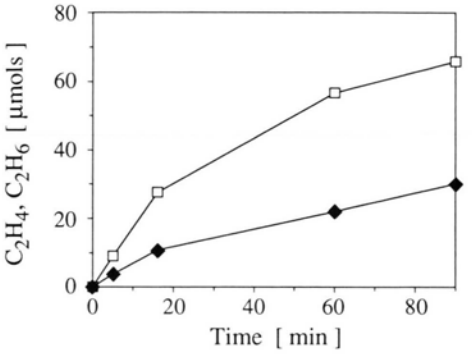


Fig. 2. Time-yield plot of the formation of C_2H_4 (□) and C_2H_6 (■) in the reduction of C_2H_2 in the Mo-nmi-hc system. Conditions as in legend to Fig. 1.

No.	System	t [min]	Yields C_2H_4 and C_2H_6 Absolute [μmol]	C_2H_4/C_2H_6	Remarks[b]
1	MoO_4^{-2}	5	0.23/0.42	0.55	het
2	1 + nmi, 1:1	5	0.09/0.016	5.63	hom
3	MoO_4^{-2}, Cys, 1:1	5	1.9/2.5	0.76	hom
4	1 + hc, 1:1	5	7.8/2.1	3.71	het
5	1, nmi, hc, 1:1:1	5	9.3/3.9	2.38	hom
6	4	10	17.0/7.5	2.26	hom
7	4, + ATP	10	32.0/5.7	5.61	hom
8	4	16	28.1/11.2	2.50	hom
9	4	60	57.1/22.0	2.59	hom
10	4	90	66.4/24.5	2.71	hom

Table I. Yields of C_2H_4 and of C_2H_6 from C_2H_2 in various systems and under different conditions[a].

[a] Reaction solutions contained 14.2 μmol of MoO_4^{-2} and the other additives at molar ratios as indicated in pH 9.6 borate buffer (0.05 F) in a total volume of 2 ml. Initial $[C_2H_2]_{initial}$ = 223 μmol; $[NaBH_4]_{initial}$ = 7 mmol; [b] het = solutions become heterogeneous; hom = solutions remain homogeneous.

ed are approximately 5 times more active than those in the molybdothiol systems. They are also more selective with respect to the formation of C_2H_4, suggesting a prevalence of mononuclear over binuclear catalytic Mo species in this system. We have previously shown [2], that the reduction of C_2H_2 to C_2H_4 involves reactive mononuclear Mo derivatives, while C_2H_6 is formed on reaction of C_2H_2 with μ-oxo bridged dimolybdenum species. Just as in the molybdothiol systems, the reduction of C_2H_2 to C_2H_4, but not to C_2H_6, is also significantly stimulated by ATP in the Mo-nmi-hc system, see Table I.

In the absence of nmi, the reaction solutions become rapidly heterogeneous due to the formation of insoluble lower-valent Mo hydroxide species. The nitrogen base evidently keeps Mo in solution. Other nitrogen bases, among them benzimidazole, can replace nmi without significant losses of catalytic activity; however, pyrazole, imidazole, pyridine and 5-hydroxyquinoline sulfate produced less active systems (Table I, Fig. 3). The mixtures of MoO_4^{-2} with nmi or other bases alone remain homogeneous but exhibit only low catalytic activity.

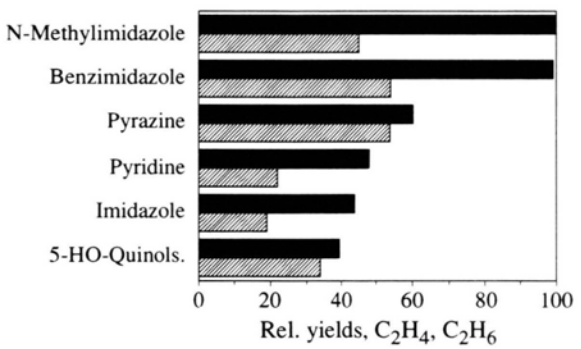


Fig. 3. Relative yields of C_2H_4 (black) and C_2H_6 (shaded) in mixtures of MoO_4^{-2} and hc with different bases, at molar ratios of 1:1:1. Conditions as in legend to Figure 1. Yields measured after 20 min of reaction. "5-HO-Quinols." is 5-hydroxyquinoline-10-sulfonate.

In C_2H_2 reduction experiments with different carboxylic acids as cofactors, *homocitric acid (hc) produced the most active catalyst* and also exhibited the highest selectivity with respect to the formation of C_2H_4. Tartaric acid produced catalysts that were almost as active, but much less selective, reducing C_2H_2 to a near 1:1 mixture of C_2H_4 and C_2H_6. Citric acid was also significantly less active and selective than hc (see Fig. 4).

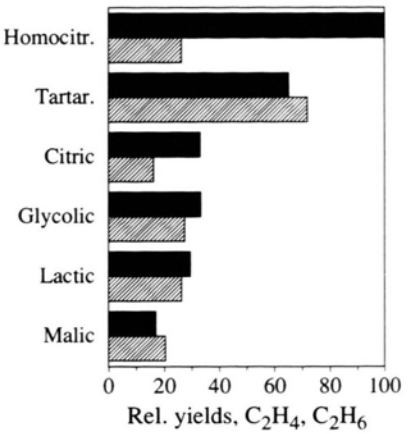


Fig. 4. Effects of different acids on the relative yields of C_2H_4 (black) and C_2H_6 (shaded) from C_2H_2 with equimolar mixtures of 14.2 μmol of MoO_4^{-2}, nmi and the respective acids under the same conditions as given in the legend to Fig. 1. Yields given relative to the yields of C_2H_4 in the Mo-nmi-hc system, measured after 20 min of reaction at 20 °C.

In the absence of nitrogen bases, the mixtures of MoO_4^{-2} with all the acids investigated turned heterogeneous under the reducing conditions, indicating a significant stabilizing effect of the nmi component. The attachment of Mo to the *his*-imidazol moiety of the FeMo-protein thus can be rationalized. Our study also clearly indicates that hc is required for maximum catalytic activity of imidazole-linked Mo species. These results thus provide further support the mechanism of nitrogenase action which invokes molybdenum as the site of substrate binding and reduction. The structural characterization of the catalytically active species in the Mo-nmi-hc- and related model systems and the reduction of nitrogen and of other substrates will be reported in forthcoming papers of the series.

Experimental Section

Reagents and chemicals

All organic compounds were commercially available and used as received. Sodium molybdate, $Na_2MoO_4 \cdot 2\,H_2O$; was analytical grade (Baker Analyzed Reagent), and used without further purification. Acetylene (Matheson) was washed with concentrated H_2SO_4; argon (National Cylinder Gas) was 99.995% and used directly from the tank.

Standard gas chromatographic technique

Hydrocarbons were measured by GLC using a Varian 1440 Aerograph laboratory gas chromatograph equipped with a 6ft phenyl-isocyanate-Porasil C, 80–100 mesh column, using FID detection. The identity of the individual gases was checked by measurements of the retention times at several operating temperatures, by coinjection and mass-spectrometry.

Standard acetylene reduction technique

The experiments were performed in reaction bottles of 38 mL capacity with rubber seals (from Pierce Chemical Co., Rockford, III.) into which measured amounts of pH 9.6, 0.1 F borate-buffered solutions of *e.g.* Na_2MoO_4, nmi and hc were added, typically at equimolar ratios, or as indicated. The bottles were flushed with argon gas at 1 atm., and 5 cm_3 of C_2H_2 at 1 atm. was injected by means of a syringe. At t = 0, 0.5 mL of a freshyl prepared 1.45 M solution of $NaBH_4$ was injected. For hydrocarbon yield measurements, gas-pressure relieve syringes of 50 cm^3 capacity were inserted into the rubber serum seals to reduce the inside pressure to 1 atm., at which the gas samples were withdrawn for hydrocarbon analysis by GLC. Detailed conditions are given in the legends to the Figures and to Table I.

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