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 FeS₈ unit from the MoFeS₈ 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 suggestion that a 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 C₃H₂ 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 pasteurianum [8] have now established that the Mo ion is attached to the histidine imidazole nitrogen atom (his-442, N=O) 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 FeS₈ unit of the FeS₈ 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, “Mo₄b(hc)-his”, would be released by way of a reversible redox-linked dissociation of the enzyme bound FeMo-cofactor-cluster, enz-FεS₈Mo(hc)-his, under the reducing conditions of the functioning enzyme, as indicated in eq. (1):

\[ \text{enz-FεS₈Mo(hc)-his} + \text{ne}^- \rightarrow \text{enz-FεS₈} + \text{Mo₄b(hc)-his} \]  \hspace{1cm} (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 FeS₈ clusters with several different heterometal ions. The generation of Mo₄b(hc)-his, which is suggested to occur in functional nitrogenase in analogy to these reactions, is shown in eq. (2):

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In accord with ref. [8] the Fe$_3$S$_4$-unit of the enz-
Fe$_7$S$_8$ cluster would act as a tridentate ligand, cap-
able of binding the oxidized form of Mo$_{\text{red}}$(hc)-his
after completion of each substrate reduction step.

The Mo atom in Mo$_{\text{red}}$(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$_7$S$_8$ unit
would serve as the electron transfer catalyst. In or-
der to obtain experimental support for this mecha-
nism, the reduction of acetylene (C$_2$H$_2$), a widely
used alternative substrate of nitrogenase, was in-
vestigated with mixtures of MoO$_4^{2-}$, he 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 en-
zymic Mo site.

Scheme I

Model System Components:

\[
\text{MoO}_4^{2-} \quad \text{CH}_2\text{CH}_2\text{CH(OH)}\text{CH}_3\text{COOH} \quad \text{CH}_2\text{N}^-\text{N}^+\text{CH} \]

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

Results and Discussion

The reduction of C$_2$H$_2$ with aqueous buffered
mixtures of Na$_2$MoO$_4$, he and nmi as catalyst precu-
sors 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$_2$H$_2$. The
most active catalysts resulted from equimolar mix-
tures of MoO$_4^{2-}$, 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$_2$H$_4$ and C$_2$H$_6$ from the reduction of
C$_2$H$_2$ with Mo-nmi-hc systems relative to the yields with
molybdocysteine catalysts, with NaBH$_4$ as the reducing
agent; [C$_2$H$_2$]$_{\text{initial}}$ = 223\,\mu\text{mol}, [NaBH$_4$]$_{\text{initial}}$ = 7\,mmol.
Reaction solutions contained 14.2\,\mu\text{mol of MoO}_4^{2-}, with
or without 14.2\,\mu\text{mol 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\,
\mu\text{mol 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$_2$H$_4$, black; C$_2$H$_6$, shaded;
rel. to yields in Mo-cys system.

<table>
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<tr>
<th>No.</th>
<th>System</th>
<th>t [min]</th>
<th>Yields C$_2$H$_4$ and C$_2$H$_6$ [\mu\text{mol}]</th>
<th>C$_2$H$_4$/C$_2$H$_6$</th>
<th>Remarks$^b$</th>
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<td>MoO$_4^{2-}$</td>
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<td>0.55</td>
<td>het</td>
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<td>5.63</td>
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<td>MoO$_4^{2-}$, Cys, 1:1</td>
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<td>hom</td>
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<tr>
<td>4</td>
<td>1 + hc, 1:1</td>
<td>5</td>
<td>7.8/2.1</td>
<td>3.71</td>
<td>het</td>
</tr>
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<td>5</td>
<td>1, nmi, he, 1:1:1</td>
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<td>9.3/3.9</td>
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<td>90</td>
<td>66.4/24.5</td>
<td>2.71</td>
<td>hom</td>
</tr>
</tbody>
</table>

Table I. Yields of C$_2$H$_4$ and of
C$_2$H$_6$ from C$_2$H$_2$ in various sys-
tems and under different condi-
tions.$^a$

$^a$ Reaction solutions con-
tained 14.2\,\mu\text{mol of MoO}_4^{2-} and
the other additives at molar ra-
tios as indicated in pH 9.6 borate
buffer (0.05\,F) in a total volume of
2 ml. Initial [C$_2$H$_2$]$_{\text{initial}}$ = 223\,
\mu\text{mol}; [NaBH$_4$]$_{\text{initial}}$ = 7\,mmol.

$^b$ het = solutions become hetero-
genous; hom = solutions re-
main homogeneous.

Fig. 2. Time-yield plot of the formation of C$_2$H$_4$ (□) and
C$_2$H$_6$ (■) in the reduction of C$_2$H$_2$ in the Mo-nmi-hc sys-
tem. Conditions as in legend to Fig. 1.
ed are approximately 5 times more active than those in the molybdothiol systems. They are also more selective with respect to the formation of C₂H₄, suggesting a prevalence of mononuclear over binuclear catalytic Mo species in this system. We have previously shown [2], that the reduction of C₂H₂ to C₂H₄ involves reactive mononuclear Mo derivatives, while C₂H₆ is formed on reaction of C₂H₂ with μ-oxo bridged dimolybdenum species. Just as in the molybdothiol systems, the reduction of C₂H₂ to C₂H₄, but not to C₂H₆, 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 MoO₄²⁻ with nmi or other bases alone remain homogeneous but exhibit only low catalytic activity.

\[
\begin{array}{|c|c|c|}
\hline
\text{N-Methylimidazole} & \text{Benzimidazole} & \text{Pyrazine} \\
\hline
\text{Pyridine} & \text{Imidazole} & \text{5-HO-Quinols.} \\
\hline
\end{array}
\]

![Fig. 3. Relative yields of C₂H₄ (black) and C₂H₆ (shaded) in mixtures of MoO₄²⁻ 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.](image)

In the absence of nitrogen bases, the mixtures of MoO₄²⁻ 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, Na₂MoO₄·2H₂O; was analytical grade (Baker Analyzed Reagent), and used without further purification. Acetylene (Matheson) was washed with concentrated H₂SO₄; argon (National Cylinder Gas) was 99.995% and used directly from the tank.

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Fig. 4. Effects of different acids on the relative yields of C₂H₄ (black) and C₂H₆ (shaded) from C₂H₂ with equimolar mixtures of 14.2 µmol of MoO₄²⁻, nmi and the respective acids under the same conditions as given in the legend to Fig. 1. Yields given relative to the yields of C₂H₄ in the Mo-nmi-hc system, measured after 20 min of reaction at 20 °C.
Standard gas chromatographic technique

Hydrocarbons were measured by GLC using a Varian 1440 Aerograph laboratory gas chromatograph equipped with a 6 ft 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, Ill.) into which measured amounts of pH 9.6, 0.1 F borate-buffered solutions of e.g. Na₂MoO₄.n H₂O and H₂O were added, typically at equimolar ratios, or as indicated. The bottles were flushed with argon gas at 1 atm., and 5 cm³ of C₂H₂ at 1 atm. was injected by means of a syringe. At t = 0, 0.5 mL of a freshly prepared 1.45 M solution of NaBH₄ was injected. For hydrocarbon yield measurements, gas-pressure relieve syringes of 50 cm³ 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.