BIOLOGICAL ACTIVITY OF SYNTHETIC MOLYBDENUM-IRON-SULPHUR, IRON-SULPHUR AND IRON-SELENIUM ANALOGUES OF FERREDOXIN-TYPE CENTRES

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Summary

The molybdenum-iron-sulphur cluster \([\text{Fe}_6\text{Mo}_2\text{S}_8(\text{SCH}_2\text{CH}_2\text{OH})_6]^{3-}\), which contains two \(\text{Fe}_3\text{MoS}_4\) cubane-like centres, is the best plausible analogue available to date for the molybdenum site of the nitrogenase enzymes. The iron-sulphur cluster \([\text{Fe}_4\text{S}_4(\text{S-C}_2\text{H}_2\text{OH})_4]^{2-}\) and the iron-selenium cluster \([\text{Fe}_4\text{Se}_4(\text{S-C}_2\text{H}_2\text{OH})_4]^{2-}\) are structural analogues of the ferredoxin \(\text{Fe}_4\text{S}_4\) active centre. All three clusters would replace ferredoxin and mediate electron transfer to \(\text{Clostridium pasteurianum}\) hydrogenase in a \(\text{H}_2\)-evolving system with sodium dithionite as the electron donor. The clusters would not replace hydrogenases which themselves are unable to evolve \(\text{H}_2\) from reduced ferredoxins. The molybdenum-iron-sulphur cluster would also replace ferredoxin in a chloroplast-ferredoxin-hydrogenase \(\text{H}_2\) evolving system.

Introduction

Recent extended X-ray absorption fine structure (EXAFS) studies on the nitrogenases from \(\text{Azotobacter vinelandii}\) and \(\text{Clostridium pasteurianum}\) and on the FeMo- cofactor of the former [1,2] have suggested that a possible structural candidate for this molybdenum site is an \([\text{Fe}_3\text{MoS}_4]\) cubane-like system. These data led us to investigate the reaction chemistry of mixtures
containing molybdenum, iron and sulphur, and subsequently to synthesise and structurally characterise complexes (I) containing two [Fe₃MoS₄] units bridged across their molybdenum atoms by three μ-thiolato-groups [3,4].

\[ \text{[NR₄]₃}[\text{Fe₆Mo}_2\text{S}_₈(SR)_₉] \quad (\text{R} = \text{Ph, Et, etc.}) \]

(I)

Although the mechanism of nitrogenase action has still to be satisfactorily elucidated, the molybdenum site and presumably, therefore, the Fe₃MoS₄ unit can be assumed (at least) to be involved in electron-transfer processes involving substrate and/or another iron-sulphur centre. Recently cyclic voltammetric [5] and differential pulse polarographic measurements (Christou, G., Garner, D. and Miller, R.M., unpublished data) in Me₂SO have shown that complexes (I) are capable of undergoing two reversible one-electron reductions. As an initial enquiry into the electron-transfer properties of these clusters we have investigated their ability to mediate electron-transfer to the hydrogenase enzyme (H₂:ferredoxin oxidoreductase, EC 1.12.7.1), in hydrogen-evolution systems, using dithionite or illuminated chloroplasts as the electron donor. The experiments were performed in aqueous solution to prevent denaturation of the enzyme and the water-soluble complex (I; R = Et, R' = CH₂CH₂OH; see Ref. 4) was employed. The earlier studies [6] of electron transfer reactions of the ferredoxin active-site analogue (NMe₄)₂[Fe₄S₄(SCH₂CH₂OH)₄] and its selenium homologue (NMe₄)₂[Fe₄Se₄(SCH₂CH₂OH)₄] have been extended.

Materials and Methods

All chemicals were of the highest available purity and were obtained from BDH Chemicals, Ltd., Poole, Dorset, U.K. unless otherwise stated. Preparation of the analogue clusters: [NEt₄]₃[Fe₆Mo₂S₈(SCH₂CH₂OH)₉]Me₂CO was prepared by ligand exchange as previously reported [4]. [NMe₄]₂[Fe₄S₄(SCH₂CH₂OH)₄] was prepared from elemental sulphur and [NMe₄]₂[Fe₄Se₄(SCH₂CH₂OH)₄] from elemental selenium using the procedure reported for the sulphur complex [7]. Stock solutions were prepared in either Me₂SO or 50 mM Tris-HCl buffer, pH 8.5; the latter solutions also contained five equivalents of mercaptoethanol per ligated HOCH₂CH₂S⁻ to prevent hydrolysis [8].

Hydrogenase and ferredoxin

These were prepared as indicated in the following references: Clostridium pasteurianum hydrogenase [9], but modified as previously described [10]; Rhodospirillum rubrum hydrogenase, [11]; Escherichia coli hydrogenase, [12]; Spirulina maxima ferredoxin, [13].

H₂-evolution assay

The 2 ml reaction mixture, containing the hydrogenase and sodium dithionite (10 mM) in 50 mM phosphate buffer, pH 7.0, was incubated under N₂ at 30°C in 15 ml sealed vials in a shaking water-bath. The clusters were added by syringe to start the reaction. Where Me₂SO was used as solvent for the Fe-Mo dimer and the Fe₄Se₄ cluster, the maximal final concentrations were 1.5% and 10% (v/v) respectively. H₂ was determined as described previously [10]. All
results are the average values from three separate experiments using two different preparations of the same cluster; for the Fe₄Se₄ cluster one preparation only was used. One unit of hydrogenase activity catalysed the evolution of 1 µmol H₂ per h with methyl viologen (2.5 mM) as the electron carrier.

\( H₂ \)-evolution from the chloroplast-hydrogenase system

This was performed as described previously [14]. In 15 ml vials, the 2 ml reaction mixture contained spinach chloroplasts equivalent to 100 µg chlorophyll, 100 nmol glucose, 20 units of glucose oxidase, 170 nmol ethanol, 1000 units of catalase, 10 mg bovine serum albumin (fat-free, fraction V) in 20 mM potassium phosphate buffer, pH 7.0, under a N₂ atmosphere at 25°C in a shaking water-bath. The system was illuminated at a light intensity of 11 000 lux. Ferredoxin or the cluster (see text) were then injected and the reaction was started by the addition of \( C. pasteuriunum \) hydrogenase (30 units). \( H₂ \) was determined as above.

Results and Discussion

The synthetic model clusters \([\text{Fe₆Mo₂S₈(S·CH₂CH₂OH)₉}]^{3⁻}, [\text{Fe₄S₄(S·CH₂-CH₂OH)₄}]^{2⁻}\) and \([\text{Fe₄Se₄(S·CH₂CH₂OH)₄}]^{2⁻}\) (henceforth termed the FeMo dimer, the Fe₄S₄ cluster and the Fe₄Se₄ cluster, respectively) were tested to see if they would replace the various components of a \( H₂ \)-evolving system.

**FeMo dimer**

The FeMo dimer was used as solution in Me₂SO (Me₂SO dimer) or in Tris-HCl buffer, pH 8.5, with added mercaptoethanol (Tris dimer, see Methods). As shown in Table I, in the absence of hydrogenase the FeMo-dimer was unable to catalyse \( H₂ \) evolution from dithionite as the electron source, with either ferredoxin or methyl viologen as the electron carrier. Thus the FeMo dimer would

<table>
<thead>
<tr>
<th>Hydrogenase</th>
<th>Electron carrier</th>
<th>( H₂ ) evolved after 10 min (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cps, Ec or Rr</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Cps</td>
<td>Fd</td>
<td>1.73</td>
</tr>
<tr>
<td>Ec or Rr</td>
<td>Fd</td>
<td>0</td>
</tr>
<tr>
<td>Cps</td>
<td>MV</td>
<td>2.93</td>
</tr>
<tr>
<td>Ec</td>
<td>MV</td>
<td>1.75</td>
</tr>
<tr>
<td>Rr</td>
<td>MV</td>
<td>1.51</td>
</tr>
<tr>
<td>Cps</td>
<td>Tris dimer</td>
<td>2.86</td>
</tr>
<tr>
<td>Cps</td>
<td>Me₂SO dimer</td>
<td>0.225</td>
</tr>
<tr>
<td>Ec</td>
<td>Tris or Me₂SO dimer</td>
<td>0</td>
</tr>
<tr>
<td>Rr</td>
<td>Tris or Me₂SO dimer</td>
<td>0</td>
</tr>
<tr>
<td>Tris or Me₂SO dimer</td>
<td>MV</td>
<td>0</td>
</tr>
<tr>
<td>Tris or Me₂SO dimer</td>
<td>Fd</td>
<td>0</td>
</tr>
</tbody>
</table>
not function as a hydrogenase: Table I also shows the ability of the FeMo dimer to act as an electron carrier to hydrogenase from dithionite. *C. pasteurianum* hydrogenase readily evolved $\text{H}_2$ from the reduced $\text{Me}_2\text{SO}$ dimer or Tris dimer but the hydrogenases of *E. coli* and *R. rubrum* would not. It may be noted that the hydrogenases of *E. coli* and *R. rubrum* are unable to use ferredoxin as an electron carrier [11,12] but readily evolve $\text{H}_2$ from dithionite-reduced methyl viologen. The FeMo dimer would thus replace ferredoxin in the $\text{H}_2$ evolving system but is not a general mediator since it would not substitute for methyl viologen. Since *C. pasteurianum* hydrogenase was obligatory for $\text{H}_2$ production in a system containing dithionite and the FeMo dimer, the $\text{H}_2$ evolved does not originate directly from the breakdown of the FeMo dimer but arises from a hydrogenase-catalysed redox reaction.

The effect of the dimer concentration on the rate of $\text{H}_2$ evolution is shown in Fig. 1. The Tris dimer was more active than the $\text{Me}_2\text{SO}$ dimer but the kinetics were the same. Both showed increasing activity with increasing concentration but between 70–200 $\mu \text{M}$, the kinetics were very unusual. At dimer concentrations above 400 $\mu \text{M}$, the Tris dimer was used instead of the $\text{Me}_2\text{SO}$ dimer to eliminate any effects of $\text{Me}_2\text{SO}$ on the enzyme activity (see Ref. 15). Fig. 2 shows that the $\text{H}_2$ evolution activity increases with increasing FeMo-dimer concentration reaching a maximum at about 2.0 mM, when the activity was similar to that obtained with saturating concentrations of ferredoxin (50 $\mu \text{M}$). The

![Graph](image1)

**Fig. 1.** $\text{H}_2$ evolution with the FeMo-dimer as the electron carrier. The 2 ml reaction mixture (see text) contained *C. pasteurianum* hydrogenase (50 $\mu \text{l}$, 350 units/ml) with the Tris-dimer (•) or the $\text{Me}_2\text{SO}$-dimer (○) as the electron carrier. The activity was calculated from the first 10 min of $\text{H}_2$ evolution.

![Graph](image2)

**Fig. 2.** Effect of Tris FeMo-dimer concentration on the rate of $\text{H}_2$ evolution. The 2 ml reaction mixture (see text) contained *C. pasteurianum* hydrogenase (50 $\mu \text{l}$, 350 units/ml) with the Tris-dimer as the electron carrier. The activity was calculated from the first 10 min of $\text{H}_2$ evolution.
apparent $K_m$ of the Tris dimer for hydrogenase (calculated as the substrate concentration giving half-maximal velocity, approx. 1.2 mM) is greater than the apparent $K_m$ value of 51 $\mu$M reported for C. pasteurianum ferredoxin but lower than that of methyl viologen (6.25 mM, [9]).

The rate of H$_2$ production with the Tris dimer as the electron carrier remained constant for less than 5 min before rapidly decreasing; only trace amounts of H$_2$ were evolved after 30 min. In contrast, the rate of H$_2$ evolution with the Me$_2$SO dimer was constant for well over 30 min. Thus, the Tris dimer was more active but less stable than the Me$_2$SO dimer. However, both dimers were much less stable than methyl viologen or ferredoxin, which support H$_2$ evolution at a constant rate for several hours. The mercaptoethanol added to the Tris dimer was not responsible for the increased activity as the activity of the Me$_2$SO dimer in the H$_2$ evolution assay was not stimulated by the presence of mercaptoethanol.

The decreased rate of H$_2$ production with the FeMo dimer as the electron carrier was assumed to arise from the instability of the dimer in aqueous solution. However, if fresh Me$_2$SO dimer, dithionite or hydrogenase were added, alone or in combination, to a reaction mixture containing the Me$_2$SO dimer, dithionite and hydrogenase after a 1-h incubation (when H$_2$ production had stopped), there was no further H$_2$ evolution. Also addition of methyl viologen after 1 h did not result in further H$_2$ evolution. Similar results were obtained with the Tris dimer. Thus, it would appear that inhibition of the hydrogenase by material arising from the decomposition of the FeMo dimer also contributes to the observed decrease in the rate of H$_2$ production.

$Fe_4S_4$ cluster

The $Fe_4S_4$ cluster was unable to evolve H$_2$ with either methyl viologen or

![Graph](image-url)

**Fig. 3.** Effect of $Fe_4S_4$- or $Fe_4Se_4$-cluster concentration on the rate of H$_2$ evolution. The 2 ml reaction mixture (see text) contained C. pasteurianum hydrogenase (50 $\mu$L, 404 units/ml) with the $Fe_4S_4$- ($\circ$) or the $Fe_4Se_4$-cluster (●) as the electron carrier. The activity was calculated from the first 10 min of H$_2$ evolution.
Fig. 4. Duration of $H_2$ evolution with the Fe$_4$S$_4$- or Fe$_4$Se$_4$-cluster as the electron carrier. The 2 ml reaction mixture (see text) contained C. pasteurianum hydrogenase (50 µl) with either methyl viologen (MV), the Fe$_4$S$_4$-cluster (Fe$_4$S$_4$) or the Fe$_4$Se$_4$-cluster (Fe$_4$Se$_4$) as the electron carrier at the indicated concentration.

ferredoxin as the electron carrier from dithionite and thus would not function as an hydrogenase. However, the Fe$_4$S$_4$ cluster in Tris solution would replace ferredoxin in the $H_2$-evolving system and mediate electron transfer to C. pasteurianum hydrogenase from dithionite, as with the same cluster used as a Me$_2$SO solution [6]. As shown in Fig. 3, saturation was observed at a cluster concentration of 3--4 mM. The initial rate of $H_2$ production was about 4-fold greater than that obtained with either methyl viologen or ferredoxin as the electron carrier (Fig. 4). The rate of $H_2$ evolution remained constant for less than 10 min though at lower concentrations of the cluster, $H_2$ production continued for a longer period (Fig. 4). Unusual kinetic behaviour was observed when low Fe$_4$S$_4$ cluster concentrations were used (Fig. 5). By comparison with previous results [6], the Fe$_4$S$_4$ cluster was more active in Me$_2$SO than in Tris solution (up to a final concentration of 0.3 mM) although the kinetics were similar. As with the FeMo dimer, the Fe$_4$S$_4$ cluster supported $H_2$-evolution for longer periods in Me$_2$SO solution than when used in Tris solution. The Fe$_4$S$_4$ cluster was also unable to mediate electron transfer to E. coli or R. rubrum hydrogenases.

Fe$_4$Se$_4$ cluster

The Fe$_4$Se$_4$ cluster was used as a solution in Me$_2$SO. The cluster would not function as a hydrogenase, nor would it act as an electron carrier to R. rubrum or E. coli hydrogenases with dithionite as the electron donor. It would however, replace ferredoxin in a $H_2$-evolving system with C. pasteurianum hydro-
The results, given in Figs. 3 and 4, show that the rates of H₂ evolution obtained with the Fe₄Se₄ cluster were slightly lower than those obtained with the Fe₄S₄ cluster and the hydrogenase was saturated at a lower concentration (about 1 mM Fe₄Se₄). It should be noted that relatively high Me₂SO concentrations were used (up to 10%, v/v). Again, low concentrations of the Fe₄Se₄ cluster supported H₂ evolution for a longer period. Fig. 5 shows that with the Fe₄Se₄ cluster, the unusual kinetic behaviour seen with the other clusters described above was not apparent.

In conclusion, all the clusters tested would replace ferredoxin in the dithionite H₂-evolving system but will not function as general bipyridyl-type redox carriers nor as hydrogenases. All three clusters will support high rates of H₂ evolution with C. pasteurianum hydrogenase, at least comparable to those obtained with ferredoxin or methyl viologen as the electron carrier. In view of the fact that when H₂ production ceases, the hydrogenase was inactivated, the observed decrease in the duration of H₂ evolution seems to arise in part from inhibition of the hydrogenase by breakdown products of the cluster.

**Photoactivated H₂ evolving systems**

Methyl viologen may be photoreduced by irradiation with visible light in the presence of proflavine with EDTA as the electron donor. If hydrogenase is also present at time zero, H₂ is evolved upon illumination of the system (see Refs. 16–18). Ferredoxin will substitute for methyl viologen in this system and will couple to C. pasteurianum hydrogenase with the evolution of H₂. As yet we have not been able to show that the FeMo dimer, the Fe₄S₄ cluster or the Fe₄Se₄ cluster can replace ferredoxin in this activated H₂-evolving system.

Ferredoxins may be reduced by illuminated chloroplasts which in the presence of hydrogenase leads to the evolution of H₂ [10,19]. Attempts to substitute ferredoxin with the Fe₄S₄ cluster or the Fe₄Se₄ cluster were unsuc-
TABLE II
REPLACEMENT OF FERREDOXIN BY THE FeMo-DIMER IN THE CHLOROPLAST-HYDROGEN-ASE SYSTEM

The 2 ml reaction mixture (see text) contained S. maxima ferredoxin or the Tris FeMo-dimer as the electron carrier at the concentration indicated.

<table>
<thead>
<tr>
<th>Electron carrier</th>
<th>Concentration (µM)</th>
<th>H₂ evolved in 1 h (µmol H₂/mg chlorophyll)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Ferredoxin</td>
<td>50</td>
<td>28.0</td>
</tr>
<tr>
<td>FeMo-dimer</td>
<td>50</td>
<td>0.7</td>
</tr>
<tr>
<td>FeMo-dimer</td>
<td>250</td>
<td>2.0</td>
</tr>
<tr>
<td>FeMo-dimer</td>
<td>500</td>
<td>8.3</td>
</tr>
<tr>
<td>FeMo-dimer</td>
<td>1000</td>
<td>11.2</td>
</tr>
</tbody>
</table>

cessful, there was no H₂ evolution. However, the FeMo dimer in Tris solution (but not in Me₂SO solution) would replace ferredoxin. As shown in Table II, the rate of H₂ production catalysed by C. pasteurianum hydrogenase increased with increasing FeMo dimer concentrations up to 1 mM. The optimal ferredoxin concentration in this system is about 50 µM [14]. We feel that the ability of the FeMo dimer to substitute for ferredoxin is an important step towards replacing the biological electron transfer components and the chloroplast-hydrogenase system with synthetic analogues.

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References


