BIOLOGICAL ACTIVITY OF SYNTHETIC TETRANUCLEAR IRON-SULPHUR ANALOGUES OF THE ACTIVE SITES OF FERREDOXINS

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SUMMARY: Two water-soluble tetranuclear iron-sulphur clusters have been synthesised which are stable under anaerobic conditions in the presence of excess thiol in aqueous solution. In such a solution, in the presence of sodium dithionite, they undergo a reversible one electron reduction to the trianion. These two clusters can replace ferredoxins in a hydrogen evolving system using Clostridium pasteurianum hydrogenase with dithionite as the electron donor; we believe this is the first demonstration of such biological activity.

INTRODUCTION Simple alkylthiolate and peptide tetranuclear iron-sulphur clusters are good models for the chemical properties of the $[Fe_4S_4(Cys)_4]$ active centres of unfolded 4Fe-4S and 8Fe-8S ferredoxins (1). We wish to report that this similarity can now be extended to include biological activity, viz. the mediation of electron transfer to hydrogenase.

In general, it is difficult to attempt the assay of the activity of analogue clusters in enzyme systems, for two principal reasons; (a) most are insoluble in water and only soluble in solvents which cause protein denaturation; (b) those clusters which are water-soluble undergo hydrolysis in water-dimethyl sulphoxide (DMSO) mixtures containing more than about 40% water. However, it has been shown that such clusters can be stable in aqueous solution in the presence of an excess of the appropriate thiol (2,3).

As a standard assay the in vitro hydrogen evolving system utilizing Spirulina maxima ferredoxin as electron mediator from dithionite to Clostridium pasteurianum hydrogenase was chosen for its simplicity and flexibility, and also because Reeves and Hall (4) have recently shown that hydrogen evolution could be obtained even in 50% DMSO. The water-soluble clusters I and II (see Methods) are stable under strictly anaerobic conditions in aqueous solution in the presence of a moderate excess of the appropriate thiol. They can substitute for ferredoxin and function as specific

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electron carriers in the hydrogen evolving system using C. pasteurianum hydrogenase as terminal acceptor and dithionite as electron donor, the details of which we report here.

MATERIALS AND METHODS

$[Fe_4S_4(Ac.Gly_2.Cys.Gly_2.Cys.Gly_2.NH_2)_2]^{2-}$

I

$[Fe_4S_4(S.CH_2.CH_2OH)_4]^{2-}$ $[Fe_4S_4(SR)_4]^{2-}$

II III

Cluster I. N-Acetyldiglycyl-S-benzyl-L-cysteinyl-diglycyl-S-benzyl-L-cysteinylglycylglycine amide (5) was debenzylated with anhydrous liquid hydrogen fluoride in the presence of anisole as carbonium-ion scavenger (6). The product (40 mg; 65.8μmol) in DMSO (1.5 ml) was treated with a solution of the bis-tetramethyl-ammonium salt of the t-butyl cluster (III, R = Bu^t) (7,8) (32.9 μmol) in DMSO (533μl) under strictly anaerobic conditions. Evaporation under reduced pressure to remove t-butyl mercaptan and final pumping out at high vacuum gave cluster I (96% yield, based on exchange with benzenethiol (9). The ^1H n.m.r. and u.v. spectra (λ_{max} 411 and 290 nm, Σ_{max} 15900 and 22400 in DMSO) were those expected for structure I, the polarographic half peak potential measured at 25°C in 80% aqueous DMSO using a vitreous carbon electrode was -0.95 V (referred to the standard calomel electrode).

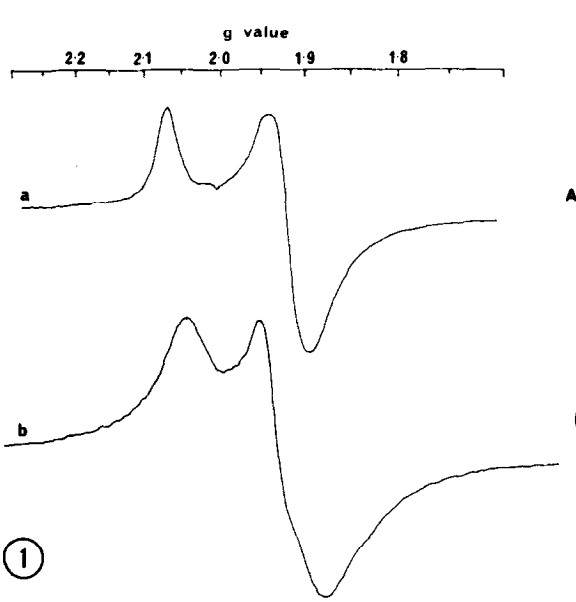
Cluster II, prepared as described by Hill et al (3), (98% yield based on exchange with benzenethiol) had λ_{max} 414 and 297 nm (Σ_{max} 16300 and 23000) in DMSO.

Electron Paramagnetic Resonance measurements and sodium dithionite reduction. E.p.r. spectra were recorded on a Varian E-4 spectrometer. Instrument settings (Fig. 1): (a), modulation amplitude, 6.3 G; modulation frequency, 100 kHz; microwave power, 5 mW; microwave frequency, 9.25 GHz; temperature, 13 K: (b) modulation aplitude, 10 G; modulation frequency, 100 kHz; microwave power, 20 mW; microwave frequency, 9.07 GHz; temperature 16 K. The cluster III (R=Ph) or I (0.3 μmol) in DMSO/0.05 molar Tris/Cl buffer (pH 8.0) 4:1) (300 μl) were reduced with dithionite at 0°C (III (R=Ph) 0.2 molar dithionite (3μl) for 25 s (spectrum a); I - 0.1 molar dithionite (8 μl) for 60 s (spectrum b) and frozen.

Electronic spectra, sodium dithionite reduction and reoxidation. Electronic spectra were recorded on a Pye-Unicam SP 800B spectrometer using 1 mm pathlength anaerobic cuvettes. Octapeptide (3.26 μmol) in 0.05 molar Tris/Cl buffer (pH 8.3) (300 μl) was treated with cluster I (0.24 μmol) in DMSO (8 μl); spectrum (a) (Fig. 2) is corrected for the absorption of the excess peptide. The cluster solution was treated with 0.12 molar dithionite (4 μl; 0.48 μmol) at 0°C and spectrum (b) recorded after 60 s. A limited amount of oxygen was introduced into the cuvette and the spectrum recorded after 25 min.

Hydrogenase and Ferredoxin. C. pasteurianum hydrogenase was prepared by the method of Chen and Mortenson (10) as modified by Rao et al (11). Rhodospirillum rubrum hydrogenase was prepared by the method of Adams and Hall (12), S. maxima ferredoxin was prepared by the method of Hall et al (13).

Hydrogen Evolution Assays. All procedures were carried out anaerobically in 15 ml vials; a 2 ml reaction mixture was used, with components as shown in the text. Addition of cluster (in 100% DMSO) gave a final DMSO



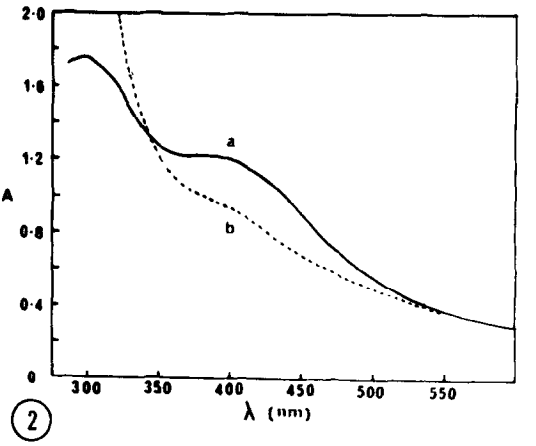


Fig. 1. Comparison of e.p.r. spectra of (a) cluster III (R=Ph) and (b) cluster I reduced with dithionite in buffered 80% DMSO. See text for details.

Fig. 2. Electronic spectra of cluster I, (a) in aqueous buffer containing excess octapeptide and (b) after the addition of dithionite at 0°C. See text for details.

concentration of approximately 0.5%. Control experiments showed this to have no effect on the hydrogenase or ferredoxin. All experiments were carried out at 30°C. The hydrogen present in the gas phase was analysed on a Taylor Servomex gas chromatograph with a "Poropak Q" column (60-80 mesh) (11).

RESULTS AND DISCUSSION The stability of clusters I and II in dilute aqueous DMSO in the presence of a moderate excess of thiol was established spectroscopically (cf. ref. 3) (peaks at 300 and 375-380 nm). Dithionite-reduced cluster I in buffered 80% DMSO gives an almost axial e.p.r. spectrum (g_{av} = 1.97: see Fig. 1b) similar to that of cluster III reduced under the same conditions (see Fig. 1a) (cf. refs 14,15). In the case of cluster II the $2^-/3^-$ couple has been characterized polarographically (3). Addition of freshly prepared aqueous sodium dithionite to a dilute aqueous solution of cluster I (2 mole per mole of cluster) at 0°C resulted in the almost complete disappearance of the peak at 375-380 nm (see Fig. 2); admission of a limited amount of oxygen regenerated the original spectrum but with a slight loss (ca. 8%) of absorbance at 375-380 nm. These

experiments demonstrate the formation of the trianion and indicate the essential reversibility of the $2^-/3^-$ couple in aqueous solution (3,16).

The two clusters were tested for their ability to replace ferredoxin and hydrogenase in a hydrogen evolution assay using dithionite as the electron donor (Table I). As can be seen, both clusters showed H_2 evolution in the presence of C. pasteurianum hydrogenase and dithionite - there was no H_2 evolution in the presence of dithionite plus standard dye mediators (no hydrogenase present). Thus the clusters appear to replace ferredoxin in the assay, but not hydrogenase. The presence of hydrogenase was obligatory for H_2 evolution, so the hydrogen does not come from breakdown of the cluster, but from a catalytic redox reaction. Various other controls shown in Table I support this interpretation. In addition more than 15 moles of hydrogen could be evolved per mole of cluster added, and the initial rate of H_2 evolution was proportional to the amount of hydrogenase added.

Hydrogenases are also capable of evolving H_2 using bipyridyl dyes (such as methyl viologen and benzyl viologen) as electron carriers. To see if the clusters were mimicking a ferredoxin or just a general redox mediator we used hydrogenase purified from R. rubrum. This hydrogenase has been shown (12) to be capable of using methyl viologen for H_2 evolution, but incapable of using ferredoxin. The two clusters would not support H_2 evolution in this system, and thus were not acting merely as bipyridyl type carriers.

The time course of H_2 evolution using optimum amounts of clusters I or II is shown in Fig. 3. The time course with saturating Spirulina ferredoxin is shown for comparison. Both clusters show linear rates of H_2 evolution for about 10-20 mins, but the rate then declines. Cluster I showed some evolution for up to 5 hours in our system, whereas H_2 evolution with cluster II ceased after 1 hour. Cluster I (at saturating concentration) showed an initial rate of H_2 evolution of about 80% of that obtainable with Spirulina ferredoxin, whereas cluster II (optimal concentration) showed an initial rate twice that obtainable with the ferredoxin.

Fig. 4 shows the effect of varying the concentration of cluster used with a standard amount of hydrogenase (the rates were calculated from the first 10 mins of linear H_2 evolution). Cluster I showed an increase in rate with increasing concentration until saturation was reached at approx. 0.8 mM. The shape of the curve for cluster II is more unusual, sigmoidal at lower concentrations, showing a decrease after a sharp optimum. In classical enzyme systems, such sigmoidal behaviour is indicative of cooperativity, but we are dubious of using such an explanation at this stage.

TABLE I HYDROGEN EVOLUTION USING CLUSTER I AND II

HYDROGENASE	CLUSTER	MEDIATOR	H_2 EVOLVED (μmoles in 1 hour)
CP	-	Ferredoxin	4.4
CP	I	-	2.4
CP	II	-	5.6
CP	-	-	0.05
CP	-	5 mM MV	7.3
-	I or II	Ferredoxin	0
-	I or II	5 mM MV	0
-	I or II	5 mM BV	0
-	I or II	5 mM MeB	0
-	I or II	-	0
RR	-	5 mM MV	2.1
RR	I or II	-	0
RR	-	Ferredoxin	0

The basic reaction mixture contained, in a final volume of 2.0 ml, 50 mM phosphate buffer pH 7.0 and 10 mM dithionite. The concentrations of cluster I, cluster II and Spirulina ferredoxin used were those found to give maximum rates of hydrogen evolution. The hydrogenases were partially purified and 10 μl were added per reaction mixture to give the rates shown.

Abbreviations used: CP, C. pasteurianum hydrogenase; RR, R. rubrum hydrogenase; MV, methyl viologen; BV, benzyl viologen; MeB, methylene blue.

The possibility of the cluster hydrolysing more rapidly in dilute solution is unlikely (17). The inhibition of the rate of hydrogen evolution at concentrations greater than 0.2 mM could be due to an impurity present or to inhibition by an hydrolysis product of the cluster. However, no impurity could be detected and mercaptoethanol up to a concentration of 10 mM had little effect on the rate or the longevity of hydrogen evolution. At the present time we have no explanation for such inhibition. Although the data obtained from the clusters was not suitable for classical enzyme kinetic analysis, for both clusters it is possible to say that the affinity of the cluster for hydrogenase (calculated from the substrate concentration at half maximal velocity in comparison with ferredoxin) is lower than that of

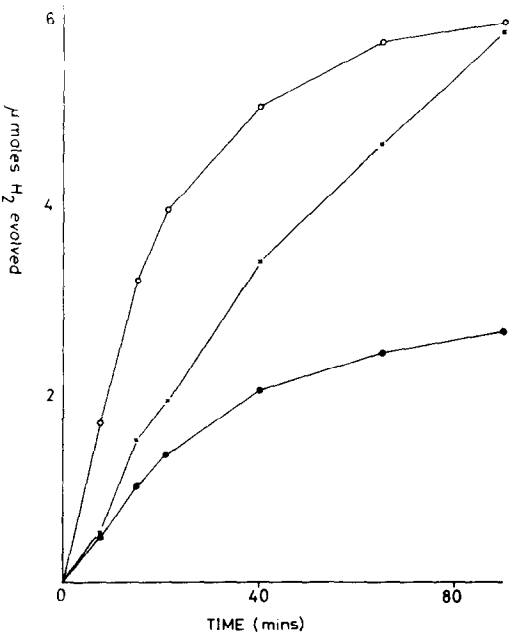


Fig. 3. Hydrogen evolution with cluster I, cluster II and S. maxima ferredoxin. (●——●), cluster I; (○——○), cluster II: (X——X) ferredoxin. The reaction mixture contained, in a volume of 2 ml, 50 mM Tris/Cl pH 7.3, 10 µl of a partially purified hydrogenase, and 10 mM $Na_2S_2O_4$. To initiate the reaction, ferredoxin (25 µM) cluster I (750 µM) or cluster II (190 µM) were added. H_2 evolution was assayed as in the text.

ferredoxin for hydrogenase, although cluster II shows a greater affinity than cluster I.

Attempts were made to increase the stability of the cluster. The addition of a low concentration of cysteine hydrochloride (approx. equimolar with the cluster present) had little effect on hydrogen evolution. However, at much higher concentrations (10 mM) significant inhibition was observed. The presence of a 2-15 fold excess of the peptide used in the synthesis of cluster I has been shown to stabilise it in aqueous solutions (cf. ref.3). We found, however, that the peptide had no effect on H_2 evolution at low concentrations, and caused inhibition at high concentrations. This is possibly due to steric effects, interfering with the binding of the cluster to the enzyme, or in the electron transfer from the cluster to the enzyme. Bovine serum albumin (BSA), a well-known protein protective reagent, caused total inhibition of hydrogen evolution with both clusters, possibly due to

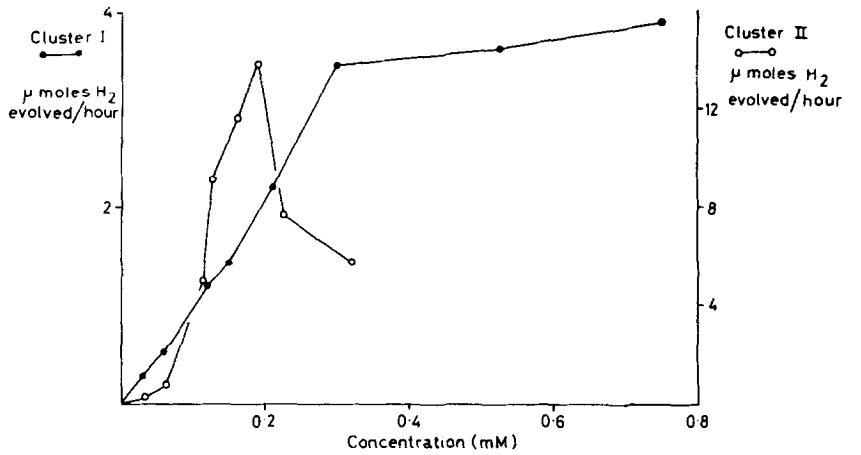


Fig. 4. Dependence of hydrogen evolution on the concentration of clusters I and II. The reaction mixture was as in Fig. 3.

adsorption of the cluster onto the BSA. As the cluster is expected to be more stable in the presence of DMSO, we studied the effects of increasing the DMSO concentration up to 40%. Previously a linear decrease in the rate of H_2 evolution using ferredoxin as mediator was found (4); this was also the case with the clusters. Presumably this is due to destruction of the hydrogenase by the DMSO.

In summary, we have synthesised two analogues of iron-sulphur centres that are reversibly reducible and partially stable in water. These analogues will replace ferredoxin as an electron mediator to bacterial hydrogenase. We believe this is the first demonstration of biological activity of iron-sulphur analogues.

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