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CONCERNING THE EXISTENCE OF SUPER-OXIDISED 8Fe-8S FERREDOXIN

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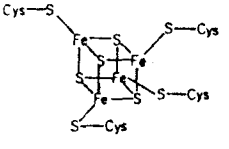
SUMMARY: Reactions of clostridial 8Fe-8S ferredoxin in the normal oxidised rest state with three inorganic oxidants have been investigated. With $IrCl_6^{2-}$ a fast reaction complete in 20s is observed. Reactions are slower with $Mn(CyDTA)^-$ and $Fe(CN)_6^{3-}$, when the extent of reaction is dependent on the amount of oxidant added (a $\sim 10^{-2}$ - fold excess was required for reactions to proceed to completion). First-order plots (Linear to 70% change) were obtained for the reaction with $Mn(CyDTA)^-$, whereas with $Fe(CN)_6^{3-}$ plots were biphasic. Spectra exhibiting the same basic features are obtained in all three cases. The product is not reduced with dithionite to normal reduced 8Fe-8S ferredoxin. The irreversible nature of the reaction, coupled with product u.v.-visible spectra, which do not resemble the spectrum of HIPIP(o), excludes a simple interpretation in terms of a third oxidation state of the protein.

INTRODUCTION: Iron-sulphur proteins are essential electron carriers in a wide range of bacterial, plant, and animal processes (1). Here we are concerned with the reactivity and accessibility of different oxidation states of 4Fe-4S clusters (2). The 4Fe-4S ferredoxins (red.pot. $\sim$ -400mV) have stable states $Fe_4S_4^{2+,1+}$. Two non-cooperative 4Fe-4S clusters $\sim 12\text{Å}$ apart (each 1-electron



active) are present in the 8Fe-8S ferredoxins. High potential 4Fe-4S proteins (HIPIP) are also known, with stable states $Fe_4S_4^{3+,2+}$ (+350mV) (3). The reasons for the different redox behaviour of the structurally near identical clusters is not fully understood (4). Oxidation of normal oxidised 8Fe-8S ferrodoxin with $Fe(CN)_6^{3-}$ to a super-oxidised form has been reported (5).

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Appendages (r), (o), (sr) or (so) indicates reduced, oxidised, super-reduced and super-oxidised states respectively.

MATERIALS AND METHODS: Clostridium pasteurianum 8Fe-8S(o) ferredoxin (M.W. ∿ 6000) was isolated as previously described (6). Absorbance peak (nm) ratios A_{390}/A_{298} = 0.79 were used as a diagnosis of purity. Protein concentrations were determined at the 390nm peak (ε 30,000 $M^{-1}cm^{-1}$ per mol of protein). A sample of Chromatium vinosium HIPIP(o) was as prepared elsewhere (7). Sodium hexachloroiridate(IV), $Na_2[IrCl_6].6H_2O$ (Johnson and Matthey), and potassium ferricyanide, $K_3[Fe(CN)_6]$, were used without further purification, peak positions nm (absorption coefficients, $M^{-1}cm^{-1}$) 487 (4075) and 420 (1010) respectively. The preparation and properties of 1,2-diaminocyclohexane-tetraacetatemanganate(III), $K[Mn(CyDTA)].2.5H_2O$, maximum 500 (345) at pH 2-7, have been described (8). Trisoxalatocobaltate (III), $K_3[Co(C_2O_4)_3].3.5H_2O$ was also prepared, peak positions 420(215) and 603(165) (9). Absorbance changes for the complexes were negligible compared to those of the protein.

Solutions of tris(hydroxymethyl)methylamine(Trizma or Tris) (0.025M), with HCl added as required, were used as buffer. At 25°C, I = 0.10M, log K_B for the protonation of Tris is 8.09. The kinetics (25°C) were followed at or near the 8Fe-8S peak at 390nm, under anaerobic conditions (N_2 gas, syringes, rubber serum cap techniques), I = 0.10M (NaCl), and pH = 7.5 except for the $Mn(CyDTA)^-$ reaction. To avoid involvement of a hydroxo form of $Mn(CyDTA)^-$ monitored at 500nm gave a rate constant 7 x $10^{-5}s^{-1}$. At pH 7.5 the aquation of $IrCl_6^{2-}$ is slow (∿ 10% in 1 hr).

Short (3m long, 0.5cm diameter) ice-cooled DEA 23 cellulose (Whatman) columns, pre-treated with degassed buffer, were used for rapid (< 10 min) separation of products. The protein was eluted with 0.5M NaCl, and made up to the original volume with buffer.

RESULTS: Concentrations of 8Fe-8S(o) protein were (1-2) x 10^{-5}M except as stated. With $Fe(CN)_6^{3-}$ the extent of reaction (30% and 95% absorbance decrease) was dependent on the amount of oxidant (2- and 10^2-fold excess respectively). First-order kinetic plots were biphasic, Figure 1. Rate constants (linear section) at different $[Fe(CN)_6^{3-}]$ were 7.4 x 10^{-4} (1.3 x 10^{-3}M) and 6.8 x $10^{-4}s^{-1}$ (5.7 x 10^{-4}M). A solution of $Fe(CN)_6^{3-}$ (6 x 10^{-4}M) and 8Fe-8S(o) (6 x 10^{-6}M) was allowed to react to completion (40 min), and the protein product separated. Relevant spectra are shown in Figure 2. On addition of dithionite (10^{-4}M) little or no change in protein absorbance was observed.

With $Mn(CyDTA)^-$ absorbance changes were again dependent on the amount of oxidant, 5% (3x excess), 25% (20x) and 80% (10^2x). First-order plots (6 x 10^{-6}M protein) were linear to > 70% completion. Rate constants were

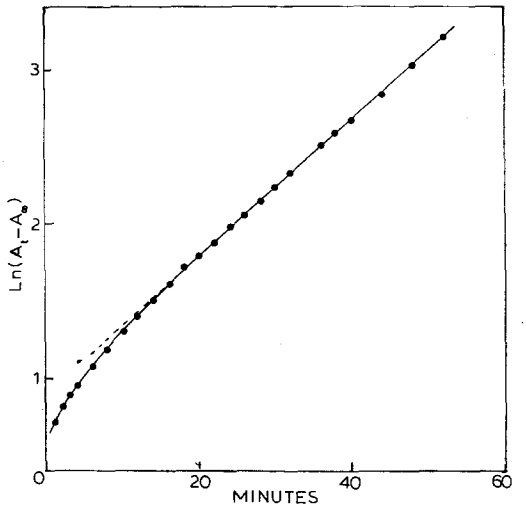

Figure 1. First-order plot of absence (A) changes, λ 390nm, against time(t) for the $Fe(CN)_6^{3-}$ (1.3×10^{-3}M) oxidation of 8Fe-8S(o) (1.4×10^{-5}M) at 25°C, pH 7.5 (Tris/HCl), I = 0.10M (NaCl).

$1.3 \times 10^{-3} s^{-1}$ (7.2×10^{-5}M complex), and $4.2 \times 10^{-3} s^{-1}$ (7.9×10^{-4}M). The latter reaction was complete in 5 min at which time the DEA separation procedure was commenced. The spectrum was recorded, Figure 2, and reactivity with dithionite tested (small absorbance decrease).

With $IrCl_6^{2-}$ as oxidant the reaction was complete in 20s. Addition of dithionite gave a slow (incomplete) decrease in absorbance over > 1h. In a further experiment with HIPIP(o) (1×10^{-5}M) and $IrCl_6^{2-}$ (1×10^{-3}M) a decrease in HIPIP absorbance λ 370nm to ∿ 80% intensity was complete in 5 min.

No absorbance change (λ 390nm) was observed over > 1h with $Co(C_2O_4)_3^{3-}$ (5×10^{-5}M) and protein (5×10^{-6}M).

DISCUSSION: Sweeney et al (5) have discussed EPR spectra of $Fe(CN)_6^{3-}$ oxidised 8Fe-8S(o) in terms of the formation of super-oxidised protein. Experiments reported here clearly indicate that more caution is required. Of the four oxidants used $Fe(CN)_6^{3-}$ (420mV) and $IrCl_6^{2-}$ (890mV) are facile redox partners in terms of inherent (kinetic) electron-transfer properties. The other two oxidants $Co(C_2O_4)_3^{3-}$ (570mV) and $Mn(CyDTA)^-$ (760mV) are less

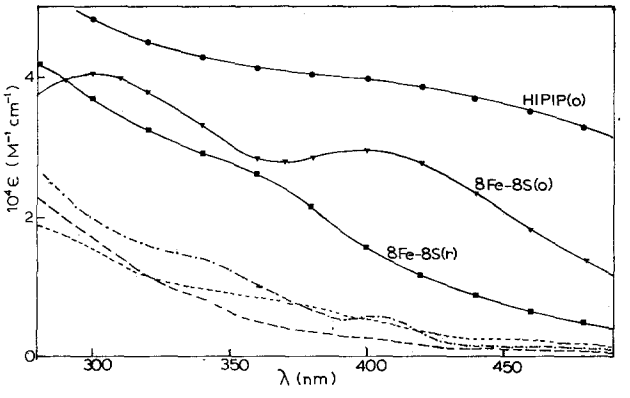

Figure 2. Spectra of 8Fe-8S(o), 8Fe-8S(r), HIPIP(o) (x2), and product spectra obtained with $Fe(CN)_6^{3-}$ (— — —), $Mn(CyDTA)^-$ (----), and $IrCl_6^{2-}$ (— · —) at pH ∿ 7.

facile. Consistent with these properties no oxidation of 8Fe-8S(o) was observed with $Co(C_2O_4)_3^{3-}$, while with $IrCl_6^{2-}$ a rapid reaction is apparent. Since $IrCl_6^{2-}$ also reacts fairly rapidly with HIPIP(o), and formation of $Fe_4S_4^{4+}$ clusters containing 4Fe(III)'s seems an unreasonable proposition, it is concluded that oxidation of the polypeptide or destructive (irreversible) oxidation of the cluster is occurring. A contrasting feature of the studies with both $Fe(CN)_6^{3-}$ and $Mn(CyDTA)^-$ is that reactions proceed to completion only when a large excess of oxidant is used. Full mechanistic implications of the rate constants presented are not at present clear to us, where more than one stage is apparent. Product spectra obtained with $Fe(CN)_6^{3-}$, $Mn(CyDTA)^-$ and $IrCl_6^{2-}$ are broadly similar, Figure 2.

Sweeney et al (5) have reported a spectrum of $Fe(CN)_6^{3-}$ oxidised 8Fe-8S(o), and comment that addition of $S_2O_4^{2-}$ gives 8Fe-8S(r). They do not state in any of their experiments how much $Fe(CN)_6^{3-}$ was used. We suggest that only ∿ 25% of their 8Fe-8S(o) reacts with $Fe(CN)_6^{3-}$. Hence substantial amounts of 8Fe-8S(o) remain for reaction with $S_2O_4^{2-}$. They have not in fact demonstrated that the reaction observed is reversible. Our experiments demonstrate conclusively that with or without DEA product separation $S_2O_4^{2-}$ is not able to effect a reduction to previously characterised states.

A feature of experiments in which HIPIP(r) is reduced with e^-_{aq}, (10) is the similarity of the spectrum generated to that of 8Fe-8S(r). This and the fact that reoxidation is possibly support the HIPIP (sr) assignment. Product spectra, Figure 1, bear no resemblance to the spectrum of HIPIP(o), and for this reason also we are reluctant to assign the 8Fe-8S(so) description. However it is of interest that some residual absorbance remains in the product spectra, and retention of some Fe/S moiety is possible.

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