Hypothesis

Thermodynamic influences on the fidelity of iron-sulphur cluster formation in proteins

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Abstract In an organism, two thermodynamic factors are important in ensuring that *homo*metallic [4Fe-4S] cubane clusters are formed in preference to clusters containing *hetero*metals such as Zn or Cu. These are the electronic resonance stabilisation, which boosts the binding of Fe(II) within an Fe-S cluster relative to its normally low position in the Irving-Williams order, and attenuation of the cytoplasmic concentrations of competing metals such as Zn or Cu by specific ligands.

Key words: Iron-sulfur; Ferredoxin; Homeostasis; Cluster

1. Introduction

Since the identification of Fe-S centres in the early 1960s, countless proteins containing these active sites have been characterised, and the structures and electronic properties of [2Fe-2S], [4Fe-4S], [3Fe-4S], [Mo7Fe-8S] and [8Fe-7S] clusters have been revealed in considerable detail [1]. Significantly, apart from the MoFe cofactor of nitrogenase (and presumably its V counterpart) or the as yet uncharacterised Ni-μX-Fe-S clusters of carbon monoxide dehydrogenase (acetyl CoA synthase), no clusters containing metals (heterometals) apart from Fe have yet been identified in isolated biological samples. The question thus arises as to whether and how Nature ensures that clusters containing only Fe are formed to the *exclusion* of those containing a heterometal. The consequences of infidelity in cluster formation could be serious. Even a single substitution, as in [M3Fe-4S] cubanes (where M is a metal that may be different from Fe) produces properties that are different from their all-Fe parents. Reduction potentials of [M3Fe-4S] hybrids may differ sufficiently from those of [4Fe-4S] and thus affect their capability for electron transfer, while gene regulatory proteins that use an Fe-S cluster as a sensor for Fe levels might respond additionally to the presence of the heterometal [2,3]. This paper addresses the underlying thermodynamic factors that favour all-Fe clusters.

2. Properties and stabilities of in vitro transformed [M3Fe-4S] clusters

The in vitro incorporation of heterometals has been achieved in several small Fe-S proteins, i.e. the ferredoxins from *Desulfovibrio gigas (Dg), Pyrococcus furiosus(Pf)* and *Desulfovibrio africanus (Da)* [4–13]. Each of these contains a cuboidal [3Fe-4S] cluster which undergoes conversion to a [M3Fe-4S] cubane adduct upon reduction in the presence of M^{2+}, as illustrated in Fig. 1. A number of model analogues have also been studied [14].

This is the simplest reaction known to interconvert Fe clusters and is possibly a key stage in the assembly and disassembly of 4Fe clusters. Certain details of this process have been determined, and enable us to assess the wider question of in vivo heterometal cluster formation.

In terms of oxidation levels, the reaction of interest is formulated according to:

$$[3Fe-4S]^{0} + M^{2+} = [M3Fe-4S]^{2+} \quad (1)$$

Binding of M^{2+} occurs to the '0' (one-electron reduced) oxidation level of the [3Fe-4S] cluster, which formally comprises two Fe(III) and one Fe(II). The [3Fe-4S] entity is thus itself a ligand, but as we will discuss, one with a elevated affinity for Fe(II). The M coordination sphere is a triad of three μ_2-bridging sulphides, with the expected tetrahedral geometry completed by a fourth ligand which varies among different proteins. Reduction potentials for $[4Fe\text{-}4S]^{2+/1+}$ clusters generally lie in the range 0 to −0.65 V vs SHE, although the '3+' level can be accessed at higher potentials (>0.3 V) in certain proteins (high-potential iron proteins) [15]. Equilibrated with a reducing intracellular environment (i.e. <0 V), the cubane product of Eq. 1 will either remain in the 2+ oxidation level, which formally comprises two trivalent and *two* divalent metals, or be reduced further. Consequently the divalent M(II) content of cubanes is always significant and Eq. 1 provides a valid basis for exploring cluster stability. Reduction potentials of [M3Fe-4S] cubane adducts formed with different M cover a wide range, suggesting that substitutions by heterometals, if they did occur in vivo, would probably interfere with redox-dependent functions [9,11,12].

The spectroscopy of [M3Fe-4S] products is well developed, and EPR, Mössbauer and NMR have led to models describing the spin coupling and electron delocalisation [10–12]. Naturally occurring heterometal clusters might be detected by their different spectroscopic signatures, for example [Zn3Fe-4S] is $S=2$ and 5/2 in its 2+ and 1+ oxidation levels (cf. 0 and 1/2 (3/2) for M = Fe) [6,10,11]. Although more extensive in vitro substitutions of Fe have been reported, products such as are found with up to all four cluster Fe atoms replaced by Cd (as with the ferredoxin from *Clostridium pasteurianum*) have not been so well characterised [16].

Stability data are available for the [M3Fe-4S] clusters in *D. africanus* ferredoxin III (Fd III) through the technique of protein film voltammetry (PFV) [9]. Adducts are weakly

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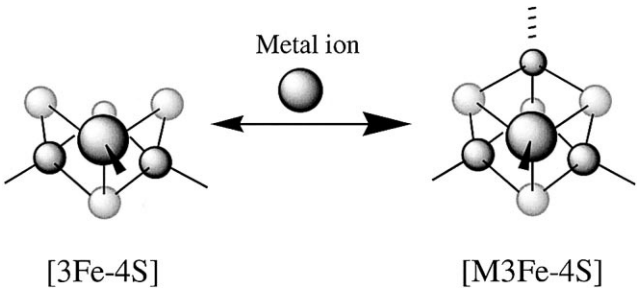

Fig. 1. Addition of a metal ion to a [3Fe-4S] cuboidal cluster to yield a [M3Fe-4S] cubane.

bound, and with the exception of Cu^{2+} (see below) reactions are rapid and reversible, greatly facilitating measurements. The ability is thus afforded to undertake experiments with precise control over the applied potential, and this enables the equilibrium described by Eq. 1 to be studied quantitatively in isolation of other interfering processes. Ferredoxin III and the ferredoxin from *P. furiosus* each have non-cysteine ligation to the labile subsite, whereas for *D. gigas* Fd a fourth cysteine is available in the appropriate position to provide the cubane with all-thiolate ligation [9,17,18]. Relevant data are shown in Table 1.

The Cu and Zn adducts are interesting since these biologically important metals clearly compete well with Fe in the in vitro experiments. The stability data for Zn, Cd, Co and Fe are quantitative, whereas the slow rate of reaction with Cu^{2+} limited its investigation beyond the conclusion that uptake is complete at levels <1 μM. Although the greater affinity for Zn vs Fe is as predicted by the Irving-Williams order, comparison with simple ligands shows that the discrimination factor is much lower than expected [19]. Fig. 2 shows the stability (association) constants log β_n ($=1/K_d$ for Eq. 1) that are obtained for complexation of M with $^{-}SCH_2CO_2^{-}$ (thioglycolate) to yield $[M(SCH_2CO_2)_2]^{2-}$ (A) and with $[3Fe\text{-}4S]^0$ to yield $[M3Fe\text{-}4S]^{2+}$ (data for *Da* Fd III). Thioglycolate provides a good comparison since it contains both S and O donor atoms, as required for the M site in *Da* Fd III, and data are available for most of the metal ions of interest [20,21]. A lower limit of log β for $[3Fe\text{-}4S]^0$ is given for Cu (which we consider for this purpose to bind as Cu^{2+}; see below) while an upper limit is given for Ni^{2+} which showed no binding at up to 1 mM concentration (note, however, that a [Ni3Fe-4S] cluster is formed with *Pf* ferredoxin [10]). Observations on *Pf* Fd also showed the $[Zn3Fe\text{-}4S]^{2+}$ cluster to be quite labile, suggesting that Zn is not so tightly held as in *Da* Fd III [11]. Although the data refer to [M3Fe-4S] clusters

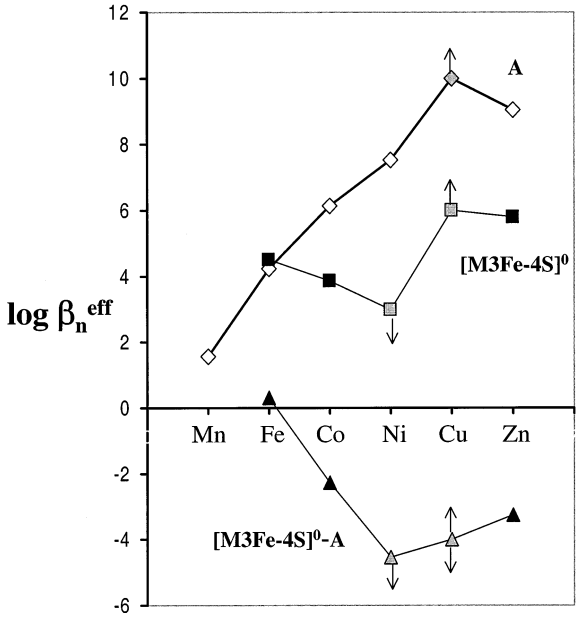

Fig. 2. Graph showing the overall binding constants β_n for various M^{2+} with the ligand $^{-}SCH_2CO_2^{-}$ ($n=2$) yielding $[M(SCH_2CO_2)_2]^{2-}$ (A), and with the $[3Fe\text{-}4S]^0$ core ($n=1$) to yield $[M3Fe\text{-}4S]^{2+}$ as measured in *Da* Fd III. The data for $[M(SCH_2CO_2)_2]^{2-}$ have been obtained from [20] but have been re-scaled to give effective β_n values at pH 7 by subtracting an increment of 6 to compensate for the high p*K*s (10) of the -SH groups in the two ligands required to form the complex [21]. The lower plot shows the difference in β_n ($[M3Fe\text{-}4S]^0-A$), revealing the advantage afforded to Fe^{2+} by incorporation in an [4Fe-4S] cluster. Arrows indicate uncertainties in values, and only a lower or upper limit can be given. The reaction of Cu^{2+} with $^{-}SCH_2CO_2^{-}$ produces Cu^{+} and sulphide, so instead a lower limit for the Cu^{2+} value has been estimated on the basis of Irving-Williams trends for other ligands.

in which M is coordinated by an O donor and not cysteine RS^{-}, it is certain (other factors being equal) that *tetrahedral all-thiolate* ligation should confer even greater enhancement in favour of Zn. The lower plot shows the difference in log β_n for the two equilibria. Whereas the plot for A follows the expected trend, it is clear from the difference plot that Fe(II) acquires a significant advantage when competing for coordination in the cluster [19].

From a thermodynamic viewpoint, the observed biological discrimination in favour of Fe, which effectively ensures that Fe-S homoclusters will dominate in vivo, is assisted by two factors: first (intrinsically) the electronic resonance stabilisation, and second (in vivo) the removal of competing metals by more strongly complexing biological ligands or by pumping.

Table 1
Equilibrium constants K_d ($=1/\beta$) for Eq. 1 as determined for *Desulfovibrio africanus* ferredoxin III, and ground spin states (*S*) observed for $[M3Fe\text{-}4S]^{(m+2)+}$ cubane adducts formed in *Da, Pf* and *Dg* ferredoxins

	Core	Adduct with						
	$[3Fe\text{-}4S]^m$	Fe(II)	Co(II)	Ni(II)	Cu(I)[a]	Zn(II)	Cd(II)	Tl(I)
K_d (μM)[b]		30	130	nd[c]	<1	1.6	0.8	1.5
S ($m=0$)	2	0	1/2	?	2	2	2	2
S ($m=1$)	1/2				1/2			1/2
S ($m=-1$)	5/2	1/2 (3/2)	1			5/2	5/2	

Data compiled from references [4–12].
[a]Reaction studied with Cu^{2+} in solution, but from spectroscopic data, Cu is bound as Cu(I).
[b]Values determined at pH 7.0, 0–4°C.
[c]Formation of a Ni adduct in *Da* Fd III has not been detected, up to a Ni^{2+} concentration of 1 mM (J.N. Butt, PhD thesis, University of California, Irvine, CA, 1993).

Table 2
The probable concentrations of free metal ions in the cytoplasm (values in mol/dm^3)

M^{2+} =	Zn	Ni	Co	Fe	Mn	Mg
Concentration[a] =	10^{-12}	10^{-11}	10^{-9}	10^{-8}	10^{-6}	10^{-3}

[a]See [19]. In each chapter on a given element the concentration of free ions is given.

We will not be concerned with the effect of kinetics in determining the selective uptake of a particular metal, but instead restrict our discussion to the thermodynamics. Many clusters such as the [Mo7Fe-8S] cluster (MoFeco) in nitrogenase are now believed to be produced under kinetic control since they require a special series of gene products [22]. Whatever the pathway by which a cluster is formed, there will remain the problem of how Fe^{2+}, a metal ion low in the Irving-Williams series, is taken up from the cytoplasm.

3. Electronic resonance stabilisation favours Fe(II) in clusters

We consider first the intrinsic resonance stabilisation. This will be expected if valence electrons from the new M(II) subsite can be delocalised over the cubane product. Another way of regarding this is that, for example, an added M(II) can acquire partial M(III) character once incorporated, and thus become more strongly bound. From Mössbauer experiments, which are conducted at temperatures down to a few Kelvin, the $[3Fe\text{-}4S]^0$ core (ground state $S = 2$) is formulated in terms of a valence-localised Fe(III) ($S = 5/2$) antiferromagnetically coupled to a ferromagnetically coupled $\langle 2Fe\text{-}2S \rangle$ pair ($S = 9/2$) with average valence 2.5 [1]. The $[4Fe\text{-}4S]^{2+}$ cluster has been well studied: it comprises two antiferromagnetic pairs of ferromagnetically coupled (delocalised) $\langle 2Fe\text{-}2S \rangle$ units ($S = 9/2$), resulting in a ground state S = 0 [1]. Thus the effect of adding Fe(II) to $[3Fe\text{-}4S]^0$ is to create a second delocalised $\langle 2Fe\text{-}2S \rangle$ ($S = 9/2$) pair. Significant stabilisation, possibly exceeding 1 eV, may be afforded relative to the situation in which the added Fe(II) remains as a valence-localised site [23]. Such energy values can raise metal ion binding affinities by more than 10 orders of magnitude. However, for M = Zn and Cd, delocalisation with the [3Fe-4S] core does not occur, and the unique Fe(III) subsite that was present in $[3Fe\text{-}4S]^0$ persists in the adduct [6,11]. These formulations are confirmed (see Table 1) by the spin states of the respective cubane products which are unchanged from the parent [3Fe-4S] cores. As expected, d^{10} metal ions are unable to create additional resonance. For M = Cu, the spectroscopy shows that the added site is Cu(I) rather than Cu(II), i.e. Cu(II) effectively oxidises the [3Fe-4S] core to the 1+ oxidation level [8,12]. In the better resolved spectra that are obtained with *Pf* Fd, the small degree of ^{63,65}Cu hyperfine coupling that is observed in the EPR spectrum of the $[Cu3Fe\text{-}4S]^{2+}$ form ($S = 1/2$) indicates only a small amount of delocalisation [12].

No such resonance stabilisation is possible for single-Fe(II) centres such as in reduced rubredoxin (Rd), which provides approximately tetrahedral, all-thiolate ligation. Indeed, recent studies show that Zn^{2+} and Cd^{2+} displace Fe(II) irreversibly from the active site [24]. Significantly however, ZnRd is not produced in the organism unless the protein is over-expressed. Metallothionein also binds Fe(II), but with an affinity that is lower than that for Cu(I) or Zn(II) [25].

4. Effective biological concentrations: Fe(II) vs competing metal ions

We next consider the in vivo situation, and examine how the concentrations of different metals depend on the buffering and pumping conditions of the cytoplasm. In particular we note that Zn/Cu sequestering proteins like metallothionein are expressed selectively, closely in accord with the amount of metal ion to which the gene is exposed [26,27]. This regulatory activity ensures that there is not much apoprotein or free metal ion in the system, and complexation reactions involving the metal ions that are highest in the Irving-Williams order are almost always close to being stoichiometric. Although exact numbers are difficult to assess, it is likely that the cytoplasmic levels of biological metal ions are similar to the values given in Table 2 [19].

Complexation of Mg^{2+} and Mn^{2+} will be dominated by O donors such as phosphates or carboxylates, but the weak binding will leave high free metal ion concentrations of approximately 10^{-3} M (Mg^{2+}) and close to 10^{-6} M (Mn^{2+}). The competition from other metal ions for these O donors is removed by the formation of almost stoichiometric complexes with stronger ligands featuring three or more donors, especially when two or more of them are N or S donors. The expected difference in binding constants between Zn^{2+} and Mn^{2+} or Mg^{2+} will then ensure that Zn^{2+} is removed from competition for the O donor centres. The same will be true of Cu^+ and Cd^{2+}, the free concentrations of which will be lowered to 10^{-12} M or below, since like Zn^{2+} they are sequestered by ligands such as metallothionein [26,27]. High-spin Fe^{2+} is not so strongly bound by the ligands generated by cells, a point exemplified by the relatively easy loss of Fe^{2+} during isolation of certain dioxygenases for which K_d probably exceeds 10^{-8} M [28]. Cellular levels of free available Fe^{2+} must depend on the prevailing electrochemical potential, with reducing conditions (< 0 V) facilitating its release from citrate and other Fe^{3+} complexes [29,30]. Note that due to the close-to-stoichiometric binding, the low residual concentrations of tightly binding free metal ions in cells becomes the inverse of the Irving-Williams series (see Table 2).

The consequence of Zn^{2+}, Cd^{2+} and Cu^{2+} (Cu^+) being targeted effectively by ligands in the cell which do not bind so strongly to Fe^{2+} is that even though proteins may well exist in which a cluster fragment such as $[3Fe\text{-}4S]^0$ has a higher absolute affinity for Zn^{2+} or Cu^+ than for Fe^{2+} (*Da* Fd III is one example) free heterometals are of such low concentration *relative* to Fe^{2+} that they cannot compete with iron in the [4Fe-4S] cluster. Similar considerations will apply in the synthesis of heterometal cluster analogues, where integration of any particular metal ion M within a [M3Fe-4S] cluster must compete with its coordination by simple ligands present in the reacting mixture [13].

The concentrations of all ions in the cytoplasm or vesicles can be controlled by pumping as well as buffering [19]. For example, levels of Ca^{2+} are held at $< 10^{-7}$ M in the cytoplasm, against concentrations of 10^{-3} M in the external environment or certain organelles. It is known that other metal ions are pumped in and out of cells or vesicles, so that their concentration can also be managed kinetically in the cytoplasm. Certainly Zn^{2+} can be as high as 10^{-4} M in some vesicles; Ni^{2+} is high (10^{-5} M) in some plant vacuoles and Mn^{2+} is $> 10^{-6}$ M in the Golgi and quite possibly in the

mitochondria and chloroplasts [19,26]. To our knowledge, Fe^{2+} is not actively pumped out of the cytoplasm and clearly we must not extend the above discussion to any compartment other than the cytoplasm without a quite different assessment of *effective* binding constants and kinetic exchange constants at the local level.

In conclusion, [4Fe-4S] clusters provide electronic resonance stabilisation of coordinated high-spin Fe(II) which is otherwise only weakly bound by biological ligands. This stabilisation and the removal of potentially competitive metal ions such as Zn^{2+} by other biological binding sites ensure the dominance of homonuclear clusters, at least in cytoplasmic proteins.

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