Electrochemical Potential and pH Dependences of [3Fe-4S] ↔ [M3Fe-4S] Cluster Transformations (M = Fe, Zn, Co, and Cd) in Ferredoxin III from *Desulfovibrio africanus* and Detection of a Cluster with M = Pb

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Abstract: The 7Fe ferredoxin from *Desulfovibrio africanus* contains a reactive 3Fe cluster which incorporates Fe and other metals to form cubanes [M3Fe-4S] having non-cysteine ligation to the added metal (M). These reactions are addressed at controlled potentials in protein molecules adsorbed on a graphite electrode, thereby facilitating an appraisal of factors that control cluster interconversions. Electrochemical and spectroscopic methods have been used to establish connectivities among interconversions with different M, explore the influence of pH, and determine reactivities of specific cluster oxidation levels. Formation of clusters with M = Fe, Zn, and Co depends on ionization of a group with pK = 5.5 + 0.3, thus supporting congruence among products and suggesting common involvement of aspartate-14 which replaces a cysteine normally present in [4Fe-4S] binding motifs. The influence of potential is complex: rapid and reversible interconversion (M = Fe, Zn) occurs only between the states $[M3Fe\text{-}4S]^{2+}$ and $[3Fe\text{-}4S]^{0}$, with $[3Fe\text{-}4S]^{1+}$ having little affinity for M. The $[M3Fe\text{-}4S]^{1+}$ cubanes and hyperreduced $[3Fe\text{-}4S]^{2-}$ are relatively unreactive. Uptake and release are significantly more rapid for M = Zn compared to Fe. Among potentially intrusive metals, Pb has a particularly high affinity for the [3Fe-4S] cluster, but the product undergoes subsequent irreversible reactions. The studies provide complementary perspectives on factors influencing cluster stability or reactivity and on the feasibility and consequences of metal substitutions.

Introduction

Known functions of iron−sulfur clusters[1] include not only dedicated electron-transfer but also acid−base catalysis[2,3] and most recently sensory action in enzyme and gene regulation.[4−8] These chemically demanding roles necessitate loss or exchange of ligands and the cluster atoms themselves. While much fundamental insight stems from studies of non-protein analogues,[9] the subtle chemistry underlying the nature and properties of clusters in proteins remains elusive despite the physiological importance of their reactions. For example, how do clusters assemble, degrade, or interconvert between different forms? To what degree is the chemistry intrinsic to cluster type rather than dictated by the protein? What are the electrochemical and pH boundaries of stability? What are the chemical feasibilities of formation of various *hetero*metal clusters? Answering these questions is aided by identifying protein systems possessing certain desirable properties, for example the presence of a cluster that is able to equilibrate very rapidly between the different species of interest and by the availability of techniques able both to examine *and* control the seemingly chaotic chemistry that often results.

Scheme 1 represents the simplest and best characterized type of interconversion between clusters of differing nuclearity and is known to occur (*in vitro* and possibly *in vivo*) in several proteins. These include aconitase[2,3] and the ferredoxins (Fd's) from *Desulfovibrio africanus* (*D.a.* Fd III),[10−15] *Desulfovibrio gigas* (*D.g.* Fd),[16] and *Pyrococcus furiosus* (*P.f.* Fd).[17−19] Coordination of M in [M3Fe-4S] may be completed by a ligand

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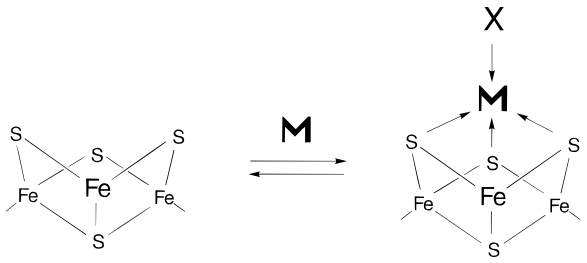
Scheme 1



(or ligands) X, which can be an amino acid or exogenous molecules such as H_2O (or OH^-). The chemistry is also elicited in non-protein analogues.[20,21]

In *D.a.* Fd III[10,22] and *P.f.* Fd[16−18,23] the [3Fe-4S] cluster is coordinated within an unconventional sequence -xaa-C-xaa-xaa-D-xaa-xaa-C-xaa in which aspartate (D) is found in the central position that would normally be occupied by one of the cysteines ligating a $[4Fe\text{-}4S]^{2+/1+}$ cluster. *D.a.* Fd III contains seven cysteines, the remaining four ligating a comparatively inert [4Fe-4S] cluster in the conventional way. It has been proposed that in *D.a.* and *P.f.* ferredoxins the labile Fe in the [Fe3Fe-4S] cluster is the one with non-cysteine ligation (thus X is not cysteine).[10,14,22,23] For *P.f.* Fd, NMR results support assignment of aspartate,[23] other possibilities being OH^- or H_2O, as for aconitase.[2,3] The [Fe3Fe-4S] cluster in both *D.a.* and *P.f.* ferredoxins binds exogenous ligands such as CN^- (*P.f.*) and thiolates (*P.f.* and *D.a.*) at the labile metal.[18,24−26]

The reaction in Scheme 1 provides a first approach to examining how cubane clusters are stabilized and alludes to the feasibility of formation of various *heterometal* clusters. For example, could clusters be formed that include "foreign" components—importantly, toxic heavy metals such as Pb? Clusters containing metals in addition to Fe have been synthesized as analogues[20,27] and are already established to have biological significance (e.g., nitrogenase).[20]

We have now extended our electrochemical and spectroscopic investigations on the formation of clusters in *D.a.* Fd III having M = Fe, Zn, Cd, Tl, and Cu.[10−15] These species appear to equilibrate rapidly with the solution environment, thus providing an excellent opportunity to view reactions and quantify cluster equilibria, although at the same time the lability poses problems for conventional investigation methods. We have used the relatively new technique of protein film voltammetry, in which a sample of protein molecules noninvasively immobilized on an electrode surface is interrogated electrochemically.[28,29] Several features serve to complement more conventional methods—i.e., sample economy with species discrimination at *pico*mole levels, sensitivity to submicromolar levels of metal ions in solution, and the ability to impose direct and precise potential control over redox centers.

Primarily, we have sought to establish reactivity relationships among [3Fe-4S] and [M3Fe-4S] clusters and define more closely the conditions for their interchange, with particular emphasis on the influences of pH (which may control the ligating capability of X) and the electrochemical potential. The potential acts by pinning particular cluster oxidation levels, thereby modulating observed equilibria and rates of interconversion. The possiblities are illustrated in Scheme 2, which shows how different Fe−S cores are linked by various individual electron-transfers and metalations.

These reactions define the stability of different core species as a function of metal ion and potential, although they neglect the influence of the protein. Reactions 8 and 9 represent, respectively, the so-called "Hi-PIP" and "ferredoxin" redox couples for [4Fe-4S] cubanes.[1] Reaction 1 is the one-electron interconversion between "1+" and "0" oxidation levels of the [3Fe-4S] cluster, whereas reactions 2 and 3 produce the intriguing species $[3Fe\text{-}4S]^{2-}$ that has recently been isolated in solution for *Sulfolobus acidocaldarius* 7Fe ferredoxin[30] and partially characterized by EPR and MCD spectroscopy. The two-electron reduction is cooperative, chemically reversible, and involves uptake of protons. The intermediate "1-" level has not been detected, although a species having this oxidation level has been generated for non-protein analogues in aprotic solvents at very low potential.[21] Due to the limited coincidence of stabilized oxidation levels, reversible interconversion between [3Fe-4S] and [M3Fe-4S] cubanes *without* electron transfer can occur only in reactions 4 or 5. Reactions 4 and 5 have been established to occur with monovalent ions (Tl)[12] and reaction 4 specifically with divalent metal-ions (Fe, Zn, Cd).[10,11,15] Entry of Cu into the system may occur by reactions 4 or 5, depending whether the metal adds as Cu(I) or Cu(II).[13]

We now appraise the connectivities of Scheme 2, reporting further cluster transformations (including lead), measurements of the pH dependence of reaction 5, and an assessment of the

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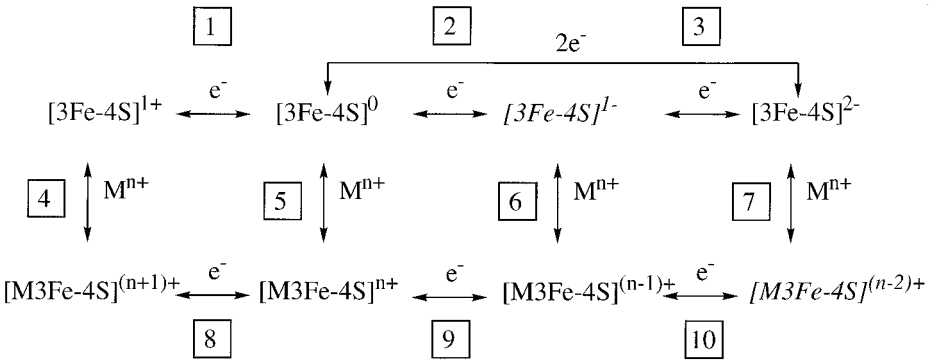
Scheme 2



reactivities of the different cluster oxidation levels. The electrochemical potential plays a very important role, and this is readily demonstrated for Fd III, a labile system with the capability to respond rapidly to changes in conditions while contained as a film on the electrode.

Methods

Ferredoxin III from *Desulfovibrio africanus*, strain Benghazi (NCIB 8401) was isolated as described[31] and stored in liquid nitrogen as concentrated beads. Concentrations were determined using $\epsilon = 29.0$ mM^{-1} cm^{-1} at 408 nm, and samples had a purity index (A_{408}/A_{280}) = 0.73.[31] Cobalt(II) chloride (6-hydrate), cobalt(II) perchlorate (6-hydrate), cadmium chloride (2.5-hydrate), cadmium perchlorate (6-hydrate), iron(II) ammonium sulfate (6-hydrate), zinc sulfate (7-hydrate), lead acetate (3-hydrate), sodium chloride, sodium perchlorate, perchloric acid, hydrochloric acid, sodium hydroxide, acetic acid, and EGTA (ethylene-bis(oxyethylenenitrilo)tetraacetic acid) were obtained from Aldrich, Johnson/Mattey, or BDH and were of at least analytical grade. Sodium dithionite was from BDH. Other reagents (Mes, Hepes, Taps, neomycin sulfate, and polymyxin B sulfate) were from Sigma. Neomycin and polymyxin B solutions were prepared as concentrated stock solutions (0.2 M or 20 mg/mL, respectively) and adjusted to pH 7.0. Purified water of resistivity ~18 MΩ.cm (Millipore or Barnstead Nanopure) was used in all experiments.

Details of electrochemical cells and the pyrolytic graphite edge (PGE) working electrode used have been described.[15,28,30] A multipot cell[28] was used for sequential transfer experiments. Experiments were carried out with an Ursar Instruments potentiostat and XY recorder (Kipp and Zonen) or an Autolab electrochemical analyzer (EcoChemie, Utrecht, The Netherlands). The saturated calomel reference electrode (SCE) was maintained at 22 °C (at which we have assumed E(SCE) is 243 mV vs standard hydrogen electrode (SHE)). Sample compartments were held at 0 °C. Reduction potentials $E^{\circ\prime}$ were determined from the average of reduction and oxidation peak potentials ($E_{pc} + E_{pa}$)/2. All values are given with reference to SHE.

For voltammetric studies, ferredoxin solutions were prepared by diluting thawed stock beads into the required buffer solutions at 4 °C and then dialyzing in an Amicon 8MC unit equipped with a microvolume assembly and a YM3 membrane. For bulk solution voltammetry and controlled potential bulk electrolysis, ferredoxin solutions contained 0.1 M NaCl buffered with 20 mM Hepes. To promote the electrochemical response, small aliquots of neomycin stock solution were added to give final concentrations 1.0−2.0 mM. For protein film experiments,[15,28] the ferredoxin solution (ca. 50−100 μM) used to coat the electrode contained 0.1 M NaCl (or 0.1 M $NaClO_4$), 20 mM Hepes, and polymyxin (typically 0.2 mg/mL, i.e., approximately 0.1 mM), or neomycin (2 mM). The pH was 7.0 at 0 °C. The buffer-electrolyte solution in the electrochemical cell consisted of 0.1 M NaCl (or 0.1 M $NaClO_4$) and 20 mM buffer (either Hepes, Mes, or a mixture of 5 mM in each of acetate, Mes, Hepes, and Taps) at 0 °C. Films were prepared by coating the freshly polished PGE electrode (chilled by standing the brass shaft in ice) with ca. 1 μL of ice-cold protein solution on the tip of a flame-drawn pasteur pipette. The electrode was then transferred promptly to the cell. To stabilize protein films, the cell solution also contained 0.2 mg/mL polymyxin or 2 mM neomycin. The pH was checked after each set of measurements.

Samples for EPR spectroscopy were prepared by electrochemical or chemical procedures. For electrochemical preparation, samples were placed in a cell featuring a pot constructed from a block of pyrolytic graphite in such a way that both the base and walls of the cavity presented an edge face to the solution.[30] Preparations made by direct electrochemical means (i.e., in the presence of neomycin) and by chemical reduction with dithionite (no neomycin) gave identical EPR spectra. EPR spectroscopy was performed on an X-band Bruker ER200-D SRC spectrometer equipped with an Oxford instruments ESR-900 helium flow cryostat.[30] Spin densities were obtained by integration of EPR absorption spectra using 1 mM Cu(II)-EDTA as standard.[32]

Results

Further Tests of Metal Ion Incorporation. As shown previously,[11−15,28] rapid and reversible binding of metals to the [3Fe-4S] cluster of Fd III is detected easily (and with considerable sensitivity) through changes in the film voltammogram occurring as the protein molecules attached to the electrode react with trace levels of metal ion in the electrolyte. Voltammetry experiments now described explore further aspects of this chemistry.

Figure 1 shows the results obtained when an adsorbed film of 7Fe Fd III is cycled at 470 mV s^{-1} in the presence of 10 μM Pb(II) at pH 6.0, 0 °C. Signals (pairs of oxidation and reduction waves) A′ and C′, associated with the couples $[3Fe\text{-}4S]^{1+/0}$ (reaction 1 of Scheme 2) and $[3Fe\text{-}4S]^{0/2-}$ (reactions 2 and 3), respectively,[30] disappear in concert with the appearance of a new signal D′ having $E^{\circ\prime}_D$ approximately −440 mV. This is only just visible, first because it overlays signals due to couple B′ (the inert $[4Fe\text{-}4S]^{2+/1+}$ cluster), and second, because a subsequent decrease in signal intensity competes with its appearance. However, the fact that a reaction has occurred is unequivocally revealed by transferring the electrode with transformed film to a buffer solution containing 20 mM EGTA (to sequester traces of Pb(II) carried over) and extending the cycle to a more oxidizing potential—a procedure known to cause release of Fe, Zn, and Cd from their respective [M3Fe-4S] clusters.[28] Signals A′ and C′ are each restored to > 50% of their expected intensity relative to B′. The Pb product is evidently unstable, since increasing the transformation time prior to cycling in buffer caused decreasing degrees of restoration of the original voltammetry.

As shown in Figure 2, cycling in 0.6 mM Co(II) causes the signals due to couples A′ and C′ to decrease in intensity and be replaced by a new signal (D′). Referring to Scheme 2, by analogy with previous work on uptake of Fe(II), Zn(II), and Cd(II), and from spectroscopic evidence to be presented below, signal D′ can be assigned to Reaction 9, the $[Co3Fe\text{-}4S]^{2+/1+}$ couple with $E^{\circ\prime}_D = -247$ mV. With Co, as with Fe, Zn, and

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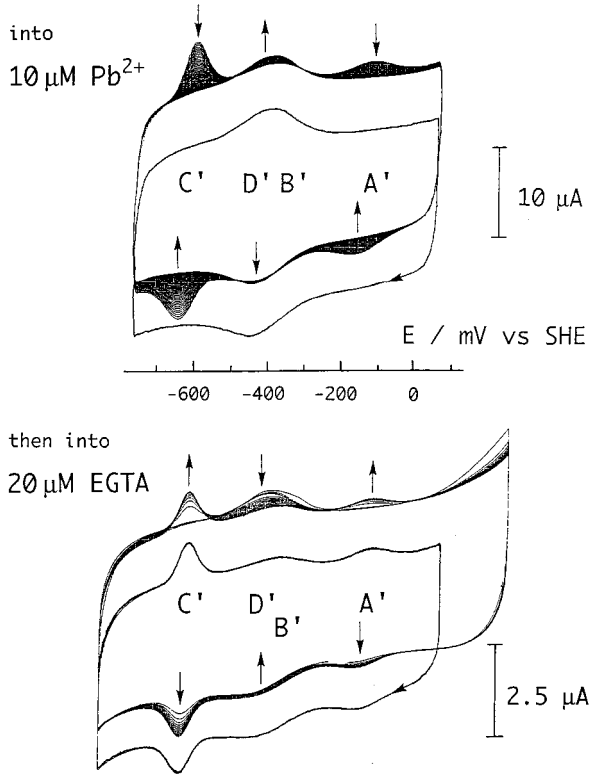


Figure 1. Upper: Successive cyclic voltammograms of a polymyxin-coadsorbed film of 7Fe Fd III at a PGE electrode and final product (single displaced trace) after transferring to a solution containing 10 μM Pb(II) (from a lead acetate stock solution) in 0.1 M NaCl, 0.2 mg mL^{-1} polymyxin, 20 mM Mes, pH 6.0, 0 °C. Scan rate 470 mV s^{-1}. Lower: Upon subsequent transfer to a solution as before but devoid of Pb(II) and containing 20 mM EGTA: successive cycles with positive switching potential increased to assist release of Pb(II); final product is shown as single displaced trace. Scan rate 95 mV s^{-1}.

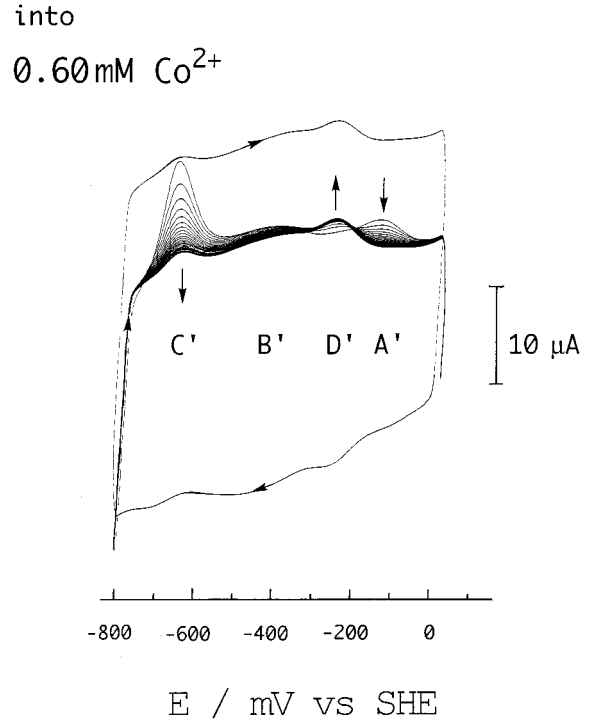


Figure 2. Cyclic voltammetry of 7Fe Fd III in a neomycin-coadsorbed film at a PGE electrode, transferred to a solution containing 0.60 mM Co(II) in mixed buffer (5 mM in each of acetate, Mes, Hepes, and Taps) pH 7.0, 2 mM neomycin, 0.1 M NaCl. Only the oxidative scans are shown in order to obtain an amplified picture of the course of reaction. The final product is shown as a displaced trace. Temperature 0 °C, scan rate 470 mV s^{-1}.

Cd,[11,15,28] the reaction was fully reversible, i.e., subsequent transfer of a transformed Fd-coated electrode to a cell containing buffer and cycling to oxidizing potentials caused reappearance of signals A′ and C′. However, even from the simple cycling procedure used, it is clear that higher concentrations of Co(II) are required to achieve a significant extent of transformation than is the case with the other metals.[33]

The equilibrium between [3Fe-4S]^0 and [Co3Fe-4S]$^{2+}$ is expressed in its simplest form by eq 1, in which C_M is the solution concentration of the metal ion M, while $C'_{[3Fe-4S]^0}$ and $C'_{[M3Fe-4S]^{2+}}$ are respective surface concentrations of [3Fe-4S]^0 and [M3Fe-4S]$^{2+}$ clusters.[11] Values for M = Fe, Cd, Zn, and

$$K_5 = \frac{C_M C'_{[3Fe-4S]^0}}{C'_{[M3Fe-4S]^{2+}}} \quad (1)$$

Tl at pH = 7.0 have been measured previously.[11,12,15] For the slower reactions (M = Fe, Cd, Zn) K_5 was determined by measuring the ratio [M3Fe-4S]/[3Fe-4S] (i.e., D′/A′) once equilibrated with various concentrations of M at fixed potentials.[11,15,28] We refer to this as the "poise-and-sample" method. For very rapidly established equilibria (e.g., Tl), dissociation constants were determined from the shift in signal A′ as a function of metal ion concentration.[12,34] The sigmoidal plot of potential vs log [Tl] yielded values for both K_4 and K_5. Similarly, we found equilibration with Zn(II) to be sufficiently rapid to use this method (employing scan rates as low as 2 mV s^{-1}) to determine an independent value for K_5 of 1 μM, compared with 1.6 μM as determined previously. Data for the [M3Fe-4S] clusters formed in Fd III and other proteins are compiled in Table 1.

To obtain further tests of the generalities and connectivities within Scheme 2, more detailed studies were carried out, and conditions influencing the uptake and release of metal ions were examined by variation of pH and applied potential. Although the complications from subsequent irreversible reactions prevented preparation of a sample of the putative [Pb3Fe-4S] cluster, solutions containing the Co adduct could be prepared and the product was investigated by EPR spectroscopy. Experiments were first undertaken to measure K_5 for [Co3Fe-4S]$^{2+}$, employing the poise-and-sample method.[11] The value obtained (130 μM) is included in Table 1.The relatively weak binding of Co(II) to [3Fe-4S]^0 combined with the small difference between the reduction potentials for [3Fe-4S]$^{1+/0}$ and [Co3Fe-4S]$^{2+/1+}$ complicated the EPR spectroscopy because it was impossible to achieve quantitative formation of [Co3Fe-4S]$^{2+}$ without using a large and interfering excess of Co(II) and applying a potential close to the region favoring [3Fe-4S]$^{1+}$.[35] Nevertheless, a sample produced with 100 μM Fd III and 95 μM Co(II) poised at −245 mV did show an EPR signal at g =

(33) A solution of 7Fe Fd III (100 μM) electrochemically one-electron reduced and treated with 1.5 equiv of Co(II) gave reversible diffusion-controlled voltammetry displaying both couples A (−140 mV) and D (−271 mV) simultaneously, thus revealing that the [3Fe-4S] cluster population is not 100% transformed. The difference (ca. 24 mV) between the $E^{\circ\prime}$ value for the [Co3Fe-4S]$^{2+/1+}$ couple in the film and in solution is somewhat greater than typically observed for this protein (<20 mV).

(34) For rapid chemical/electrochemical equilibration of species around a square such as defined by reactions 1, 5, 8, and 4, the following equation relates the observed reduction potential ($E^{\circ\prime}_{obs}$) to the reduction potential for the [3Fe-4S]$^{1+/0}$ couple ($E_1{}^{\circ\prime}$), the metal ion concentration C_M and the respective dissociation constants K_4 and K_5 for [M3Fe-4S]$^{(n+1)+}$ and [M3Fe-4S]$^{n+}$.

$$E^{\circ\prime}{}_{obs} = E^{\circ\prime}{}_1 + \frac{2.3RT}{F}\log\left\{\frac{(1 + C_M/K_5)}{(1 + C_M/K_4)}\right\}$$

Table 1. Reduction Potentials and Equilibrium (Dissociation) Constants for [M3Fe-4S] Clusters in Proteins and Analogues[k]

		M =								
host	[3Fe−4S]	Mn	Fe	Co	Ni	Cu	Zn	Cd	Tl	Pb
D.a. Fd III[a]	−140		−400	−271		n.m.	−480	−580	n.m.	n.m.
	(−150)		(−393)	(−247)		(+148)	(−492)	(−569)	(80)	(−440)
			30	*130*		*<1*	*1.6*	*0.8*	*1.5*	*<<1*
									34 × 10^{3} [b]	
	[11, 22]		[10,11]	this work		[13]	[11]	[11]	[12][c]	this work[d]
D.g. Fd[e]	−130		−420	−245	−360		n.m.	−495		
	[16c]		[16c]	[16c]	[16c]			[16c]		
P.f. Fd[f]	−160	>−100	−345	−163	>−100		−241			
	[19]	[19]	[17a, 19]	[19]	[19]		[19]			
$[MFe_3S_4(X)(SR)_3]$[g]	−548		−1008[h]	−778	−658				n.m.	
			−958[i]							
	[21b][j]		[21b, 27][h,i]	[27][h]	[27][h]					

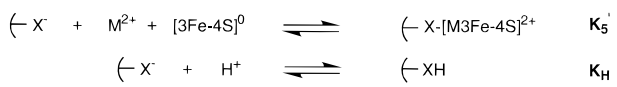
[a] Data are for pH 7.0, 0.1 M NaCl except where stated. [b] Reaction 4, K_4. [c] pH 7.0, 0.5 M NaCl. [d] pH 6.0, 0.1 M NaCl. [e] pH 7.6 values determined by potentiometric EPR titrations or square-wave voltammetry. [f] pH 8.0, values determined by potentiometric EPR titrations. [g] Values determined by cyclic voltammetry in acetonitrile, corrected to the SHE scale by adding 242 mV. [h] $(SR)_3 = LS_3$ a tripodal cavitand ligand, (X) = *N*-methylimidodiacetate. [i] SR = X = *S*-mesitylthiolate. [j] (SR) = (LS_3), no X. [k] Potentials for [M3Fe-4S] are given are for the 2+/1+ redox couple, which in Scheme 2 corresponds to reaction 7 except for M = Cu, Tl, in which case the half-cell process is reaction 8. Values for [3Fe-4S] are for the 1+/0 couple. Potentials in parentheses are $E^{\circ\prime}$ values measured for protein films. Numbers in italics are dissociation constants, K_5 except where indicated.

1.84 which disappeared as the potential was lowered. The *g*-value is similar to that observed for the $[Co3Fe\text{-}4S]^{2+}$ cluster formed in *D. gigas* Fd[16a] and non-protein clusters[27,36] and is attributed to an $S = 1/2$ ground state. However, no hyperfine coupling with Co ($I = 7/2$) was resolved, thus contrasting with the well-resolved spectra observed for $[Co3Fe\text{-}4S]^{1+}$ in *P.f.* Fd[19] and *D.g.* Fd[16a] and reflecting the equally contrasting spectra of respective $[Tl3Fe\text{-}4S]^{2+}$ clusters.[12,18c] In *P.f.* Fd,[18c] the spectrum displays resolved hyperfine coupling to Tl ($I = 1/2$), whereas, again, this is poorly resolved in Fd III.

The Influence of pH on Cubane Formation. No 3-D structures have yet been obtained for any of the various forms of *D.a.* Fd III. In particular, while there is overwhelming evidence that the metal−ion adducts are cubane-type [M3Fe-4S] clusters, the mode of noncluster ligation to the labile metal atom in each case is unknown. This ligation is likely to be provided by the aspartate (D14) carboxylate, as recently determined for the ferredoxin from *Pyrococcus furiosus*,[23] or by OH^- as established for resting-state aconitase.[3] It is also possible that the coordination number increases, as occurs for aconitase during turnover.[2,3] Each of these coordination modes should result in pH-dependent metal ion binding since −COOH or H_2O alone are poor donors (although H_2O could be stabilized by hydrogen bonding to the carboxylate, similar to substrate-bound aconitase[2]). The pH dependence of K_5 was thus examined for various metals in order to ascertain whether their binding to $[3Fe\text{-}4S]^0$ could involve a common ionizable group (X in Scheme 1).

If X is a protein ligand and coordination to M competes with its protonation, the formation of [M3Fe-4S] cubanes becomes pH-dependent, with equilibria described and defined as shown in Scheme 3 .

Scheme 3



The pH dependences of equilibria with Fe, Zn, Cd, and Co were determined by measuring K_5 at lower pH values using the poise-and-sample method with several modifications. First (to counter possible complexation of the M subsite by Cl^-) $NaClO_4$ was used as supporting electrolyte instead of NaCl, and ClO_4^- salts of the metal ions were used in several cases. Second, an interstage transfer, into a pot containing no EGTA, was employed to minimize contamination of the M(II) solution by EGTA carried over from the initial equilibration solution. Third, the voltammetric scan range used to sample cluster populations was restricted to that required to observe the A′ couple.

A decrease in the stability of clusters with M = Fe, Zn, and Co at lower pH was immediately evident because higher concentrations of M(II) were required to effect changes in the voltammogram of the 7Fe form. Results were analyzed for each metal by fitting complete datasets to a model based upon direct competition between M(II) and H^+ for ligand X. This procedure yielded values for K_H (the intrinsic H^+/X^- dissociation constant) and K_5' (the intrinsic $M(II)/X^-$ dissociation constant) noting that $K_5' = K_5$ at pH values >> pK_H (i.e., $-\log K_H$). Normalized values of K_5^{-1} (K'_5/K_5) were then used to derive the stability/pH plot shown in Figure 3. Results for M = Fe, Zn, and Co are consistent with competitive binding to a common ligand X having a p$K_H = 5.5 \pm 0.3$, with K_5' values effectively identical to determinations made at pH 7.0. By contrast, the data for Cd lie off the curve suggesting that a different ligation system is utilized. No significant differences were noted between results obtained with 0.1 M NaCl or 0.1 M $NaClO_4$. Values of $E_D^{\circ\prime}$ for the [M3Fe-4S] clusters showed only minor variations with pH, the dependence being approximately −5 mV /pH unit in each case.

The two immediate options are (a) direct ligation by the carboxylate of aspartate-14[23] or (b) ligation by a water molecule that is stabilized by the aspartate carboxylate, the latter situation resembling substrate-bound aconitase.[2] The p*K* value is similar to that assigned to aspartate (p*K* 5.4) in the 7Fe ferredoxin from *Azotobacter vinelandii*.[37,38] A third possibility which cannot

(35) The EPR spectrum of Fd III (100 μM) fully reduced at −605 mV in the presence of 0.95 equiv of Co(II) was dominated by resonances at $g = 2.05$, 1.93, and 1.88, accounting for 0.9 spins/protein molecule and assigned to the inert indigenous $[4Fe\text{-}4S]^{1+}$ cluster (see ref 22) An analogous sample electrolyzed to equilibrium at −355 mV (at which >90% of the indigenous 4Fe clusters are oxidized ($S = 0$) while most of the Co adduct should be reduced) showed weak signals at $g = 5.3$ and 2.4 (assigned to noncluster Co(II) by reference to a spectrum of Co(II)EGTA), a feature at $g = 1.93$ attributed to the remaining small fraction of indigenous $[4Fe\text{-}4S]^{1+}$ species, and a small signal at $g = 1.83$ assigned to traces of oxidized Co adduct. At −245 mV, which corresponds to <50% oxidized Co adduct, the $g = 1.83$ signal intensified, confirming a paramagnetic ground state for the 2+ level, but showing no hyperfine structure. Finally, at −205 mV there was significant reappearance of the $g = 2.01$ spectrum of $[3Fe\text{-}4S]^{1+}$, and signals at $g = 5.3$, 2.4 due to Co(II)EGTA.

(36) Roth, E. K. H.; Greneche, J. M.; Jordanov, J. *J. Chem. Soc., Chem. Commun.* **1991,** 105.

(37) Cheng, H.; Grohmann, K.; Sweeney, W. V. *J. Biol. Chem.* **1990,** *265,* 12388−12392.

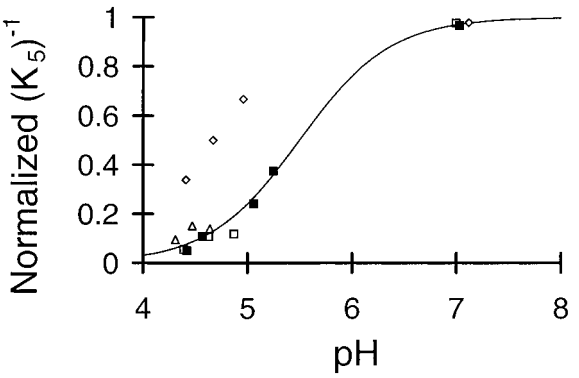


Figure 3. The pH dependence of the stability of [M3Fe-4S] clusters in *Desulfovibrio africanus* Fd III. Ordinate shows reciprocal K_5 data, $(K_5)^{-1}$, normalized to pH 7 values, i.e., $K_5^{(pH7)}/K_5$ ($\simeq K_5'/K_5$) for metal ions Fe(II) (n), Zn(II) (p), Co(II) (D) and Cd(II) (G), each measured in 0.1 M $NaClO_4$. The curve is the best fit obtained assuming the model defined in Scheme 3 and described in the text.

be discounted at present is competitive binding of a proton at or very close to the $[3Fe\text{-}4S]^0$ cluster, as is now known to occur with the ferredoxins from *Azotobacter vinelandii* and *Sulfolobus acidocaldarius*.[38−41] However, the reduction potential of signal A′, the $[3Fe\text{-}4S]^{1+/0}$ couple (i.e., reaction 1) is relatively insensitive to pH at values above pH 5.[30]

Control of Cluster Speciation by the Electrochemical Potential. Scheme 2 predicts that the binding of M within the [M3Fe-4S] cubane depends upon cluster oxidation level and consequently upon the applied potential. For M(II), rapid equilibration without electron transfer occurs only via reaction 5, for which equilibrium constants K_5 have been determined. Experiments were now undertaken to determine reactivities of the cluster species prevailing at different potentials.

A comparison was made of the M-uptake reactivities of the [3Fe-4S] cluster in its various oxidation levels. Studies were carried out with M = Zn and Fe, but for practical reasons efforts were focused on the Zn experiments since the signal D′ corresponding to $[Zn3Fe\text{-}4S]^{2+/1+}$ provides a characteristic marker (*), being sufficiently separated (ca. 0.1 V) from that of the inert [4Fe-4S] cluster. After forming a film of the 7Fe form, the electrode was transferred to a pot containing 0.5 μM Zn(II), pH 7.0, with various values of applied potential maintained during the operation, and then held for 1 min. The extent of transformation was then determined by cycling at 500 mV s^{-1} which is sufficiently rapid to minimize changes in cluster population. Typical results are shown in Figure 4. Little uptake of Zn(II) is observed after 1 min at potentials above −100 mV (−43 mV), i.e., at which [3Fe-4S] exists as the 1+ level, whereas at a potential (−457 mV) favoring both $[3Fe\text{-}4S]^0$ and the product $[Zn3Fe\text{-}4S]^{2+}$, rapid conversion occurs as noted previously. Then, as the potential is lowered below −750 mV, i.e., to "lock" the [3Fe-4S] core in the 2- level, there is again little formation of [Zn3Fe-4S]. Some attenuation of signals A′ and C′ is observed at this potential, but this is due to loss of the [3Fe-4S] cluster in this highly reduced state; a similar decrease is observed in buffer alone.[42] To test that the remaining [3Fe-4S] cluster population was still capable of metal ion uptake

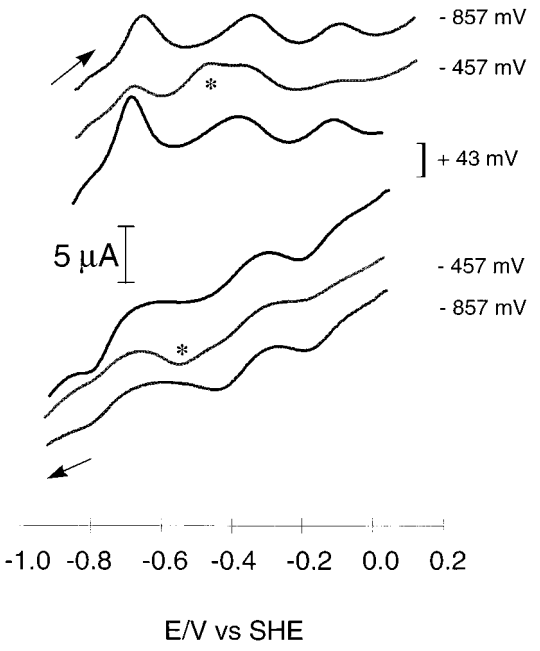


Figure 4. Cyclic voltammograms showing the extent of uptake of Zn(II) into the [3Fe-4S] cluster at different potentials. The cell solution contains 20 mM Hepes, 0.1 M NaCl, pH 7.0 at 0 °C. Films of the 7Fe Fd III were poised for 60 s at the potentials indicated, and then a cycle was recorded at a scan rate of 500 mV s^{-1}. The signal due to $[Zn3Fe\text{-}4S]^{2+/1+}$ is indicated *. The capacitance background has been largely removed to save space.

after being held at these low potentials, the film was repoised at −550 mV whereupon signals due to $[Zn3Fe\text{-}4S]^{2+/1+}$ appeared as expected. Results obtained for Fe uptake (10 μM) were similar, although less definitive because the $[Fe3Fe\text{-}4S]^{2+/1+}$ signal overlays that due to the inert $[4Fe\text{-}4S]^{2+/1+}$ cluster.

Release of M from [M3Fe-4S] to yield the [3Fe-4S] core was studied as a function of potential in analogous fashion to the uptake experiments. This involved preparing a film containing [M3Fe-4S] by cycling in a solution containing M(II) $\gg K_5$, then transferring (with the electrode connected and potential applied) to a solution devoid of M(II), poising at that potential for 1 min, and then cycling at 500 mV s^{-1} to view the status of clusters. The presence of [3Fe-4S] was revealed by the sharp, characteristic oxidation peak due to the two-electron C′ couple (reactions 2 + 3). Voltammograms shown in Figure 5 are for M = Fe. To compare reactivities, the signal C′ oxidation peak current was measured and normalized with respect to the amplitude obtained upon complete transformation. Figure 6 shows the resulting activity/potential profiles obtained for M = Fe and Zn, together, for reference, with a potential-phase diagram showing the relevent cluster oxidation levels. Considering first Fe, release is barely detectable at potentials below −400 mV where [Fe3Fe-4S] exists in the 1+ level (although there was marginal evidence for a slight increase in activity at very negative potentials, corresponding to formation of $[3Fe\text{-}4S]^{2-}$ as product). As the potential is raised above −400 mV, the extent of Fe release increases and becomes effectively instantaneous at 200 mV. No additional voltammetric signals (such as might correspond to the $[Fe3Fe\text{-}4S]^{3+/2+}$ (HiPIP) couple were observed in scans (500 mV s^{-1}) made at potentials up

(38) Shen B.; Martin, L. L.; Butt, J. N.; Armstrong, F. A.; Stout, C. D.; Jensen, G. M.; Stephens, P. J.; La Mar, G. N.; Gorst, C. M.; Burgess, B. K. *J. Biol. Chem.* **1993,** *268,* 25928−25939.

(39) Armstrong, F. A.; George, S. J.; Thomson, A. J.; Yates, M. G. *FEBS Lett.* **1988,** *234,* 107−110.

(40) Hu, Z.; Jollie, D.; Burgess, B. K.; Stephens, P. J.; Münck, E.; *Biochemistry* **1994,** *33,* 14475−14485.

(41) Breton, J. L.; Duff, J. L. C.; Butt, J. N.; Armstrong, F. A.; George, S. J.; Pétillot, Y.; Forest, E.; Schäfer, G.; Thomson, A. J. *Eur. J. Biochem.* **1995,** *233,* 937−946.

(42) Prolonged poising (several minutes) of a film of Fd III at potentials more negative than signal C′, i.e., at which the [3Fe-4S] cluster is in the hyper-reduced 2- oxidation level, results in loss of signals A′ and C′ relative to B′. This attenuation is not reversed either by cycling in EGTA or CN^- and is believed to be due to dissociation of the core from the cluster binding ligands. The $[3Fe\text{-}4S]^{2-}$ state formed in other proteins, e.g., *Sulfolobus acidocaldarius* Fd is more stable.

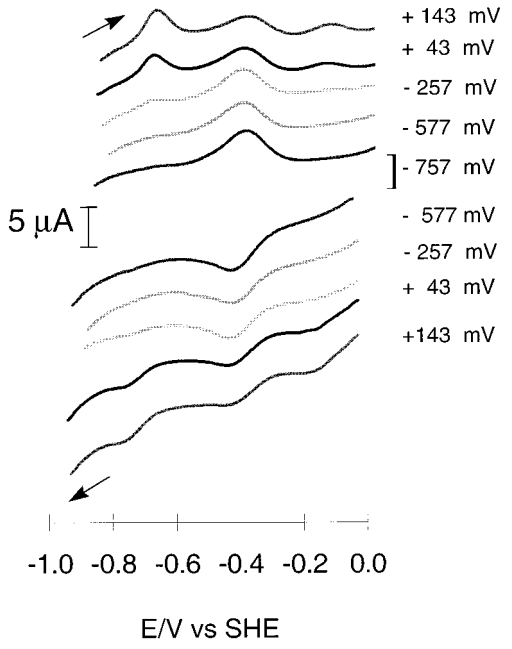


Figure 5. Cyclic voltammograms showing ejection of Fe from the [Fe3Fe-4S] cluster at different potentials, as evidenced from the appearance of signals A′ (3Fe-4S $^{1+/0}$) and C′ (3Fe-4S]$^{0/2-}$). Films of the transformed 8Fe Fd III were poised for 60 s at the potentials indicated and then cycled at 500 mV s^{-1}. Conditions: 20 mM mixed buffer, 0.1 M NaCl, pH 7.0 at 0 °C.

to 500 mV. As before, checks were made to confirm that [3Fe-4S] was produced as expected upon poising at potentials > 0 mV. Release of Zn from [Zn3Fe-4S] was more rapid, accelerating noticeably above −500 mV and then again, marginally, above −100 mV. The presence of EGTA made no difference to results obtained with Fe, although more reproducible data were obtained for Zn.[43]

Experiments were carried out to measure direct competition between Fe and Zn. Although Zn binds more tightly than Fe in reaction 5, the reduction potential of the $[Zn3Fe\text{-}4S]^{2+/1+}$ couple (−492 mV) is sufficiently more negative than $[Fe3Fe\text{-}4S]^{2+/1+}$ (−393 mV) that application of an electrode potential midway between $[Fe3Fe\text{-}4S]^{2+/1+}$ and $[Zn3Fe\text{-}4S]^{2+/1+}$ ought to produce a noticeably greater proportion of the Fe adduct since this alone would be in the 1+ state.[44] To test this idea, a film of the 7Fe Fd III was poised at either −307 or −457 mV in a solution at pH 7.0 containing the mixture 10 μM Fe(II) and 0.5 μM Zn(II), approximately in the ratio of their K_5 values. Although a greater extent of overall transformation was apparent at −457 mV, reliable quantitation of the contributions of different species could not be achieved in this congested region of the voltammogram. However, qualitatively, the results obtained at −457 mV did indicate, as expected, a relatively lower contribution of the more characteristic D′ signal due to $[Zn3Fe\text{-}4S]^{2+/1+}$. Use of higher Fe levels attenuated the $[Zn3Fe\text{-}4S]^{2+/1+}$ signal at either potential.

(43) The presence of EGTA in M-release experiments is probably more important for metal ions that bind very tightly to the cluster, thereby necessitating sequestration of even trace contaminating levels.

(44) Obviously our experiments probe reactivity (i.e., reaction within a certain time frame) rather than the positions of equilibria between states of Scheme 2 (which could be slow to establish in certain cases). Since the reduction potential for the one-electron reaction 2 is expected to be very negative (at least more negative than for reaction 9), simple thermodynamic reasoning based on the core chemistry alone predicts that $K_6 < K_5$, i.e., that M(II) is bound more tightly in $[M3Fe\text{-}4S]^{1+}$ than $[M3Fe\text{-}4S]^{2+}$. However, poise and sample experiments to test for any increased uptake when poising at potentials favoring the 1+ oxidation level did not produce conclusive results. Stabilities in this negative potential region are likely to be influenced by the participation of $[3Fe\text{-}4S]^{2-}$.

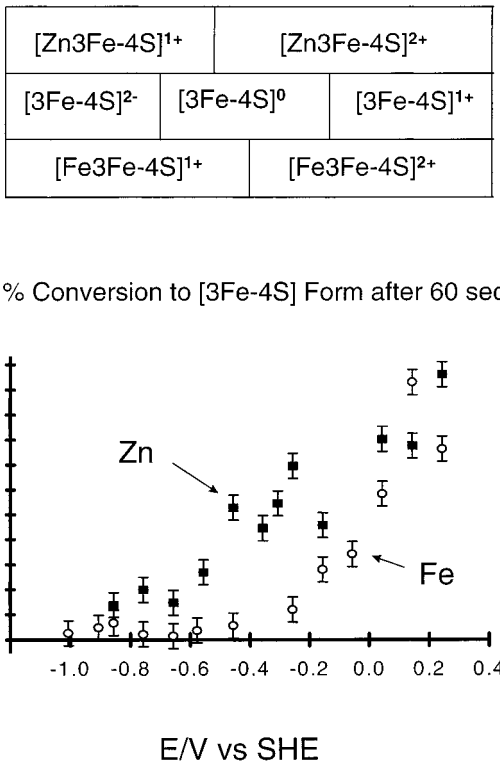


Figure 6. Potential dependence of the release of Fe and Zn from respective [M3Fe-4S] clusters in Fd III. Experiments were carried out as given in legend to Figure 5. Open symbols show results for [Fe3Fe-4S], comparing the fractional extents of reappearance of [3Fe-4S] cluster (measured from the oxidation peak for signal C′) after 60 s at the potentials indicated, with the full conversion level established after holding at 240 mV for 1 then 2 min to confirm complete reaction. Closed symbols show the analogous data obtained for [Zn3Fe-4S]. Above the graph is shown the potential phase diagram indicating the correspondence with potential ranges for existence of relevant oxidation levels of [Zn3Fe-4S], [3Fe-4S], and [Fe3Fe-4S] clusters.

General Discussion

As described below, cluster interconversions as depicted in Scheme 1 occur in a simple and reversible manner only via reaction 5. This process occurs rapidly for Fd III adsorbed as a film at PGE, showing that only a small barrier needs to be overcome to accommodate the metal ion. Rates depend on M, with Tl(I) (which also forms a weak complex with $[3Fe\text{-}4S]^{1+}$; reaction 4) displaying particularly rapid kinetics and equilibrating within a scan rate of 470 mV s^{-1}. The M(II) ions are slower, with estimated rates[11] following the approximate order, Cd(II) > Zn(II) > Fe(II) > Co(II), as expected on the basis of typical ligand substitution rates (e.g., exchange of inner-sphere water molecules).[45] Equilibration with Zn(II) was confirmed to be measurable by the method used for rapid equilibrium with Tl(I) but with much slower scan rates (1−2 mV s^{-1}). Ferredoxin III is a rather labile protein which loses entire clusters readily, thus implying considerable conformational flexibility. Other ferredoxins for which transformation products are well characterized are the single-cluster systems *Desulfovibrio gigas* Fd and *Pyrococcus furiosus* Fd.[16−19,23] These proteins each contain disulfide bridges,[46] although it is unlikely that Fd III is more reactive for this reason because it possesses instead a [4Fe-4S] cluster as cross-link. However, Fd III contains a

(45) See, for example: Cotton, F. A.; Wilkinson, G. *Advanced Inorganic Chemistry*, 5th ed.; 1988.

(46) Macedo, A. L.; Moura, I.; Surerus, K. K.; Papaefthymiou, V.; Liu, M.-Y.; LeGall, J.; Münck, E.; Moura, J. J. G. *J. Biol. Chem.* **1994,** *269,* 8052−8058: Gorst, C. M.; Zhou, Z. H.; Ma, K.; Teng, Q.; Howard, J. B.; Adams, M. W. W.; La Mar, G. N. *Biochemistry* **1995,** *34,* 8788−8795.

glutamate in place of proline that normally follows the remote cysteine:[11,31] this proline may enforce greater rigidity in the cluster domain. Conformational flexibility is also suggested by the lack of hyperfine structure in the EPR spectra of $[Tl3Fe\text{-}4S]^{1+}$ and $[Co3Fe\text{-}4S]^{2+}$, consistent with greater M-subsite heterogeneity.

The similar pH dependences of reaction 5 with Fe, Zn, and Co are consistent with metal−proton competition for a common ligand. With a pK of 5.5 (± 0.3) this is likely to be the carboxylate from aspartate (D14), as recently confirmed by NMR for *Pyrococcus furiosus* Fd,[23] although a H_2O molecule stabilized by H-bonding to aspartate is equally probable, and aspartate and H_2O could even bind simultaneously in a five-coordinate subsite. It is likely (though by no means definite) that the same ligation persists in the reduced cubanes $[M3Fe\text{-}4S]^{1+}$, since any redox-coupled ligand exchange activity would probably be revealed by complicated scan rate dependence of the $[M3Fe\text{-}4S]^{2+/1+}$ signal.[26] Another possibility which we are currently unable to eliminate is that the pH-dependence stems from direct competition with proton binding *at the cluster*. The $[3Fe\text{-}4S]^0$ centers in ferredoxins from *Azotobacter vinelandii* (Fd I, and the D15N mutant of Fd I), *Azotobacter chroococcum*, and *Sulfolobus acidocaldarius* each bind a single H^+ at or in the very close vicinity of the cluster to produce a spectroscopically characteristic species.[38−41] The pK values vary between 7.8 (*A.v.* and *A.c.* Fds I),[38,39] 6.9 (*A.v.* D15N Fd I),[29c,38] and 5.8 (*S.a.* Fd).[41] Identical measurements with Fd III indicate a pK at the lower value of 4.8, although the nature of the protonated form has yet to be established.[30]

The following situation is thus being established for Fd III: (i) the protein offers little resistance to cluster transformations, (ii) direct potential control and sensitivity is achieved through the protein film method, and (iii) the common pH dependence supports structural congruence (at least among clusters with M = Fe, Zn, and Co). These features enable us to observe and deconvolute some of the tangled relationships between cluster composition and the electrochemical potential imposed by the environment. Importantly, the rapid responses to changes in potential suggest strongly that the transformations occurring in this particular protein reflect *intrinsic* properties of the cluster species, rather than extrinsic (protein structure based) factors such as steric hindrance or inappropriate positioning of X.

For M(II) uptake, no significant reactivity is observed either for $[3Fe\text{-}4S]^{1+}$ or the hyper-reduced species $[3Fe\text{-}4S]^{2-}$. The former is too electron-deficient to coordinate a fourth divalent metal, whereas the all-Fe(II) cluster is likely to be extensively protonated.[30] For M(II) release, we observed that degradation of $[Fe3Fe\text{-}4S]^{2+}$ to produce $[3Fe\text{-}4S]^0$ was clearly visible after 1 min, while release of Zn from $[Zn3Fe\text{-}4S]^{2+}$ was significantly faster, with 50% transformation after 1 min. In both cases the rate increases further as the potential is raised above −100 mV, i.e., where the product is $[3Fe\text{-}4S]^{1+}$. Sensitivity to the nature of the product is expected for a process in which the transition state most resembles the product, which in this case has little affinity for the leaving M. Although rates of release did increase somewhat as the potential was raised further, i.e., to 200 mV, no evidence (i.e., a voltammetric wave) was obtained for any $[M3Fe\text{-}4S]^{3+}$ intermediate. The superoxidation pathway (reactions 8 and 4) is thought to be the primary route for degradation of [4Fe-4S] to [3Fe-4S] clusters, for example, by oxidation with $Fe(CN)_6^{3-}$.[47] Our experiments show that reaction 5 alone (or particularly when the product is trapped as $[3Fe\text{-}4S]^{1+}$ via reaction 1) also affords a simple pathway for cluster degradation. By contrast, the [M3Fe-4S] core is conspicuously *least* labile[44] in the 1+ level (i.e., < −400 mV for M = Fe, < −500 mV for M = Zn). Significantly, this inertness extends to the low-potential region favoring the existence of hyperreduced $[3Fe\text{-}4S]^{2-}$, a species which exhibits little activity in the *uptake* direction. The conclusion is that one or indeed both of these cluster species, $[M3Fe\text{-}4S]^{1+}$ and $[3Fe\text{-}4S]^{2-}$, are inert (kinetically stable) with regard to interconversion. The greater stability of $[Zn3Fe\text{-}4S]^{2+}$ versus $[Fe3Fe\text{-}4S]^{2+}$ (K_5 values) clearly contrasts with its relative lability but is in accordance with the higher Zn uptake rates.

As evident from Table 1, comparisons of the reduction potentials of the [3Fe-4S] clusters and their corresponding [M3Fe-4S] products in different ferredoxins serve to highlight the remarkable influence of just a single subsite—i.e., the M−X component. Notably, there is great variation among cubanes with different M, whereas the [3Fe-4S] clusters have similar potentials in all the examples given. With the exception of M = Mn and Ni for *P.f.* Fd, reduction potentials for $[M3Fe\text{-}4S]^{2+/1+}$ are always more negative than $[3Fe\text{-}4S]^{1+/0}$ despite the more positive core charge and therefore reflect the importance of local structure and intrinsic electronic factors. Several points are apparent with regard to the complex influences of X. First, we reported previously[26] that ligation by exogenous thiolate (RS^-) causes the *D.a.* Fd III $[Fe3Fe\text{-}4S]^{2+/1+}$ reduction potential to decrease by nearly 200 mV due to the much tighter (and more rapid) binding of RS^- to the oxidized cluster, although it was not possible to determine whether RS^- substitutes or adds at the cluster. Second, for M = Fe and Zn, the order of $E^{\circ\prime}$ values is reversed between Fd III and *P.f.* Fd, suggesting different M−X coordination environments in the two proteins, as recently proposed by Johnson and co-workers.[19] It is probable that many thermodynamic and kinetic differences exist among cubane M-adducts formed in different proteins, reflecting either direct changes localized in the M-subsite region or structural constraints originating elsewhere but transmitted to this region. Third, and most obviously, the lability of the non-cysteine ligated metal in these proteins and aconitase[2] stems from the replacement of cysteine by oxygen-atom donors. Several observations provide strong support for this. Thiolate RS^- is a superior ligand for late transition- and post-transition metals, and competition with protonation is much more favorable (compare the corresponding K_5' values likely for typical S-ligation, which must be $\ll 10^{-8}$ since the pK for thiol/thiolate equilibrium in free cysteine is 8.4[48]). Holm and co-workers exploited weak ligation ($X = CF_3SO_3^-$) to one unique subsite of $[Fe_4S_4(LS_3)(X)]^{2-}$ to obtain the $[3Fe\text{-}4S]^0$ analogue $[Fe_3S_4(LS_3)]^{3-}$.[21] Cowan and co-workers have reported on a mutant of HiPIP in which one cysteine is replaced by serine, the major effect of which is to render the oxidized cluster $[4Fe\text{-}4S]^{3+}$ much more prone to degradation, with evidence for $[3Fe\text{-}4S]^{1+}$ as an intermediate.[49] We also recently reported[14] that replacement of aspartate-14 by cysteine in Fd III stabilizes the [M3Fe-4S] cluster (M = Fe) with respect to [3Fe-4S] (decrease in K_5). By contrast, for *D.g.* ferredoxin and other systems with all-cysteine ligation, subsite lability must be determined by protein structure. We speculate that thiophilic metals such as Cd(II) or Tl(I) might override any need for protein ligation, instead bonding to the exposed trigonal tri-μ_2-sulfido face of the [3Fe-4S] cluster and recruiting solvent H_2O molecules to complete a coordination shell.

The revelation that [3Fe-4S] clusters interact strongly with Pb(II) has plausible consequences in terms of toxicity. It is likely, although not proven, that the initial product is a

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[Pb3Fe-4S] cluster analogous to the other cubanes and generated readily with Pb(II) levels as low as 10^{-6} M. Thus in the event of failure of adequate metal ion homeostasis, a threat might be imposed to normal Fe−S assembly in proteins and indeed at certain levels of cluster sensory activity. On the other hand, the interesting possibility arises that systems such as transcription factor FNR which may operate[8] by redox-linked [3Fe-4S] ↔ [4Fe-4S] interconversion could instead utilize other metals as surrogates to effect the desired alteration in nucleic acid binding.

The problem of examining the possibility of heterometal clusters existing in Nature, as raised by ourselves[11] and others,[18b] divides into two parts: (a) their detection and (b) their stabilities and reactivities which are intimately linked to the electrochemical potential. The latter factor in particular requires careful consideration when dealing with labile clusters vascillating between different forms. Our results suggest how certain cluster types, particularly in labile proteins, might escape detection entirely. This difficulty is apparent from our unsuccessful attempts to isolate a pure sample of $[Co3Fe\text{-}4S]^{2+}$, the main reason being that 100% formation of this weakly-bound species requires a considerable excess of Co(II). Structural sensitivity to the ambient electrochemical potential within biologically feasible limits presents a natural asset to be exploited by regulatory Fe proteins, including the Iron Regulatory Protein[4] and (most obviously) redox sensors such as transcription factor FNR.[8] The ability to monitor and control very reactive cluster species by sensitive methods such as protein film voltammetry represents an important aspect in investigations.

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Supporting Information Available: EPR spectra of Co adduct and competition experiment with Zn and Fe 0.5 and 10 e^{-06} M (2 pages). See any current masthead page for ordering and Internet access instructions.

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