Characterization of the Fundamental Protein Ligand Requirements of [4Fe-4S]$^{2+/+}$ Clusters with Sixteen Amino Acid Maquettes

Stephen E. Mulholland, Brian R. Gibney, Francese Rabanal, and P. Leslie Dutton*

Contribution from The Johnson Research Foundation, Department of Biochemistry and Biophysics, and the School of Medicine, The University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received April 15, 1998. Revised Manuscript Received August 17, 1998

Abstract: A survey of ferredoxin maquettes derived from natural sequences was utilized to obtain a primary sequence competent for [4Fe-4S]$^{2+/+}$ incorporation for the study of the minimal ligand requirements for cluster assembly. The resultant 16 amino acid ferredoxin maquette (FdM), $\text{NH}_2$-KLCEGGCIAACGACGGW-CONH$_2$, incorporates a single [4Fe-4S]$^{2+/+}$ cluster as evidenced by a cluster titration assayed by electron paramagnetic resonance (EPR) spectroscopy. Assembly of the [4Fe-4S] cluster within FdM was kinetically facile (minutes time scale) and the cluster was stable in solution under strictly anaerobic conditions. The four cysteines of FdM were systematically replaced with nonligating alanine residues resulting in lower yields (10–56% relative to a FdM control) as quantitated in the reduced state by EPR spectroscopy demonstrating the necessity for each in successful cluster incorporation. A single example of a cysteine to leucine modification resulted in a lower yield of cluster incorporation equivalent to the analogous alanine replacement indicating a general absence of steric hindrance for [4Fe-4S] assembly in these small peptides. Pairwise replacement of cysteines with alanines resulted in dramatic loss of yield and cofactor induced assembly of a pair of peptides as evidenced by EPR spectroscopy and size exclusion chromatography, respectively. Alanine substitution of three of the four cysteines from the FdM sequence resulted in a virtual loss of [4Fe-4S] incorporation ability. Attempts were made to provide non-cysteine ligands to the incorporated cluster using aspartic acid and histidine residues; however, the lower yield of [4Fe-4S] assembly in these peptides coupled with both the identical EPR spectral parameters and redox potential indicates a lack of observable aspartate or histidine ligation to the clusters. The measured yields of [4Fe-4S] incorporation provide a convenient tool for probing the basic ligand requirements for the [4Fe-4S]$^{2+/+}$ cluster in these 16 amino acid ferredoxin maquettes.

Introduction

The iron sulfur protein family is quickly being realized to be as ubiquitous and functionally diverse as the well-recognized heme protein family.$^1$ Iron sulfur proteins are widely dispersed in the Archaea, Prokarea, Eukaryotes as well as in higher organisms.$^2$ While functionally most notably associated with respiratory and photosynthetic electron-transfer complexes (for example NADH-ubiquinone oxidoreductase$^3$, Photosystem I$^4$), the iron sulfur protein family has been found to be involved in oxygen sensoring (SoxR,$^5$ FNR$^6$), dehydratase reactions (aconitase$^7$) including branched chain amino acid biosynthesis (isopropylmalate isomerase)$^8$, and iron heme protein family. Iron sulfur proteins are widely dispersed as ubiquitous and functionally diverse as the well-recognized heme protein family. For example NADH-ubiquinone oxidoreductase$^3$, Photosystem I$^4$), the iron sulfur protein family has been found to be involved in oxygen sensoring (SoxR, FNR$^6$), dehydratase reactions (aconitase$^7$) including branched chain amino acid biosynthesis (isopropylmalate isomerase)$^8$, and iron heme protein family. While the majority of iron sulfur proteins utilize cysteine residues to ligate mononuclear, binuclear, trinuclear, or tetranuclear clusters, histidine ligation is utilized in natural [4Fe-4S]$^{2+/+}$ and Rieske-type [2Fe-2S] proteins, with serine and aspartate ligation observed in natural and site-directed ferredoxin or high-potential iron protein (HiPIP) mutants.$^{13,14}$

The multifaceted complexity of natural proteins can potentially be reduced to a tractable level allowing systematic study of a variety of biochemical processes by employing maquettes$^{15}$ derived from the minimalist hierarchical approach pioneered by DeGrado and co-workers.$^{16}$ Maquettes are minimal synthetic proteins designed to provide a framework to answer particular structural, functional, and chemical questions concerning highly complex systems. They provide a tractable level of complexity that allows a systematic study of the role of specific residues in the overall function of the protein. The iron sulfur protein family is a good example of a complex system that has been studied using maquettes.

* Address correspondence to this author at the Department of Biochemistry and Biophysics.

complex native proteins. Thus, the prototype ferredoxin maquette, called FdM, demonstrated assembly of the [4Fe-4S]$_{2/2+}$ cluster in a protein module that could be combined with a heme containing four-$\alpha$-helix bundle, thereby breaking down the engineering complexities inherent in natural proteins.\textsuperscript{17} Designed from a consensus sequence of bacterial ferredoxins, the primary structure of FdM contains the CIACGAC core amino acids taken directly from Peptococcus aerogenes ferredoxin\textsuperscript{18} as shown in Figure 1. Unstructured in the absence of the bound cofactor, FdM binds a single [4Fe-4S]$_{2/2+}$ cluster under physiological conditions as evidenced by spectral characteristics (EPR g-values, line shape, and relaxation properties; UV—visible and CD spectroscopy) and measured reduction potential ($E_{\text{red}} = -350\text{mV}$) which are reminiscent of natural [4Fe-4S]$_{2/2+}$ ferredoxins.

While maquettes have been shown to offer an attractive approach to the study of protein—protein assembly,\textsuperscript{21–24} protein—cofactor incorporation,\textsuperscript{25–26} modulation of bound cofactors,\textsuperscript{15} and multicofactor assembly,\textsuperscript{27–28} herein we utilize ferredoxin maquettes to specifically probe the fundamental ligand requirements for [4Fe-4S]$_{2/2+}$ cluster binding in a 16 amino acid synthetic peptide. The validity of the prototype maquette as a template for this further study was evaluated by comparison to maquettes derived from other related natural sequences. The results begin to reveal the protein engineering specifications requisite for biochemical [4Fe-4S] cofactor assembly and confirm our design of FdM as a modular protein design unit.

**Materials and Methods**

**Chemicals and Solvents.** Iron(III) chloride, sodium sulfide, diethyl ether, $\beta$-mercaptoethanol, and trifluoroacetic acid were obtained from Aldrich Chemical Co. Ethanedithiol and 1-hydroxybenzotriazole (HOBt) were purchased from Fluka. Fmoc-protected amino acid perfluorophenyl esters were purchased from PerSeptive Biosystems except for Fmoc-L-Arg(Pmc)-OPfp which was obtained from Bachem. All other chemicals and solvents were reagent grade.

**Peptide Synthesis.** The peptides were synthesized on a continuous flow Milligen 9050 or PerSeptive Biosystems Pioneer solid phase synthesizer using standard Fmoc/Bu protection strategy\textsuperscript{29} as previously described.\textsuperscript{17a}

**Solution Molecular Weight Determination.** Gel permeation chromatography was performed anaerobically in a Plas-Labs glovebox using a Beckman System Gold HPLC with diode array detector fitted with a Superdex 30 column (Pharmacia) eluted at 2 mL/min. The eluting buffer (10 mM K$_2$PO$_4$, pH 7.0) was deoxygenated using successive freeze—pump—thaw cycles. The column was standardized using aprotinin (6.5 kDa), glutathione (0.4 kDa), apo-FdM-1,2,3A (1.3 kDa), and the homodimer of FdM-1,2,3A (2.6 kDa).

**[4Fe-4S]$_{2/2+}$ Assembly.** A standard reconstitution protocol based on modified biochemical literature methods\textsuperscript{30} was utilized to incorporate the [4Fe-4S] cluster into each of the designed peptides. Under strictly anaerobic conditions, a solution of 2% v/v $\beta$-mercaptoethanol in 50 mM HEPES buffer (pH 8) containing 100 $\mu$M peptide was equilibrated

---

**Figure 1.** Molecular model of the prototype ferredoxin maquette, FdM, and the X-ray structure of Peptococcus aerogenes ferredoxin I (PDB accession code 1FDX) on which the FdM design was based. The indicated C$_\alpha$ trace highlights the CIACGAC core motif of *P. aerogenes* Fd used in FdM.
for 2 h to reduce any adventitious disulfides. A 50 mM solution of FeCl₃ was slowly added to a final concentration of 600 μM before addition of a 50 mM solution of Na₂S to a total concentration of 600 μM (i.e. 1.5 equiv of [4Fe-4S] cluster per peptide). Each peptide was reconstituted at the same time as a sample of FdM, utilized as a control. After equilibration, the resulting iron—sulfur containing peptides were stored frozen in liquid nitrogen.

**Cluster Titration into FdM.** The addition of equimolar amounts of iron and sulfide salts to the FdM peptide resulted in an increase in bound cluster up to one cluster per peptide (four iron and four sulfur per peptide) (supplementary Figure S1). Addition of cluster beyond the first equivalent resulted in no further increase in EPR signal intensity. The slightly lower EPR spectral area upon addition of excess iron and sulfide salts, 2.0 equiv or greater, is attributed to protein precipitation. Other substituted FdM peptides displayed similar characteristics, with a rise in signal up to the measured reconstitution yield, followed by a drop in signal as the excess iron and sulfur salts began to precipitate.

**Cluster Stability in FdM.** The addition of 4 equiv of iron and sulfide salts to 100 μM FdM peptide resulted in the facile formation of the [4Fe-4S] cluster (supplementary Figure S1). After addition, aliquots were removed from the reaction mixture in a anaerobic chamber throughout all operations, reduced with a minimal amount of sodium dithionite, and frozen in EPR tubes. The EPR spectral area of the [4Fe-4S]⁺ cluster is constant for the hour it was monitored.

**UV—Visible Spectroscopy.** UV–vis spectra were recorded on a Perkin-Elmer Lambda 2 spectrophotometer using quartz cells of 0.2 and 1.0 cm path length. Peptide concentrations were determined spectrophotometrically using ε₁₀₀ = 5600 M⁻¹ cm⁻¹ for Ttr.

**EPR Spectroscopy.** Electron paramagnetic resonance (EPR) spectroscopy was performed using a Bruker ESP300E spectrometer with the frequency measured by a Hewlett-Packard 5350B frequency counter. Temperature control was maintained by an Oxford ESR 900 continuous flow cryostat interfaced with an Oxford ETC4 temperature controller. Typical EPR parameters: sample temperature, 10 K; microwave frequency 9.449 GHz; microwave power, 1 mW; modulation frequency, 100 kHz; modulation amplitude, 20.0 G; time constant, 164 ms. Power saturation determinations at 10 K were done at microwave powers between 25 μW and 200 mW. The temperature dependence of the [4Fe-4S]⁺ signals was measured at 1 mW incident power between 4 and 50 K. Unless otherwise noted, protein concentrations were 100 μM. The concentration of unpaired spins per peptide (based on tryptophan absorbance) was determined from a calibration curve based on Cu(II)-EDTA. The yields listed in Table 1 represent the average three or more reconstitutions relative to the FdM control peptide and successive reconstitutions suggest an error of approximately 10% in the given values.

**Redox Potentiometry.** Chemical redox titrations³¹ were performed in a cuvette within an inert atmosphere glovebox with platinum working and calomel reference electrodes. Ambient redox potentials (measured against the standard hydrogen electrode) were adjusted by addition of aliquots (≤1 μL) of sodium dithionite or potassium ferricyanide. Titrations were performed in 50 mM HEPES, pH 8.0, 100 mM KCl. Electrode—solution mediation was facilitated by the following mediators at 10 μM concentration: neutral red, 2-hydroxy-1,4-naphthoquinone, safranine T, and phenosafranine. After equilibration at each potential, a 300 μL sample was removed and frozen in liquid nitrogen and the EPR spectrum was recorded. The doubly integrated spectral area was plotted against potential and the data were fit to the Nernst equation with n = 1.0.

**Results**

**Survey of [4Fe-4S] Peptides Designed from Natural Sequences.** A series of maquettes derived from various iron sulfur protein natural sequences were designed to compare with our prototype ferredoxin maquette, FdM, to determine the optimal sequence for a small peptide that would competently bind a cuboidal [4Fe-4S]²⁺/³⁺ cluster. The FdM maquette has

<table>
<thead>
<tr>
<th>Table 1. Sequences of the Iron—Sulfur Maquettes in This Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maquette</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>FdM</td>
</tr>
<tr>
<td>FdM-Pa</td>
</tr>
<tr>
<td>FdM-Sp</td>
</tr>
<tr>
<td>FdM-Sokk</td>
</tr>
<tr>
<td>FdM-Kieske</td>
</tr>
<tr>
<td>FdM-1A</td>
</tr>
<tr>
<td>FdM-2A</td>
</tr>
<tr>
<td>FdM-3A</td>
</tr>
<tr>
<td>FdM-3L</td>
</tr>
<tr>
<td>FdM-4A</td>
</tr>
<tr>
<td>FdM-2.3A</td>
</tr>
<tr>
<td>FdM-1.4A</td>
</tr>
<tr>
<td>FdM-1U</td>
</tr>
<tr>
<td>FdM-2D</td>
</tr>
<tr>
<td>FdM-3D</td>
</tr>
<tr>
<td>FdM-4D</td>
</tr>
<tr>
<td>FdM-1H</td>
</tr>
<tr>
<td>FdM-1.4H</td>
</tr>
</tbody>
</table>

*The core CIACGAC motif taken from__P. aerogenes__ is between the bullets. All of the peptides are C-terminally amidated.*

at its core the typical CXXCXXC motif of bacterial ferredoxins (Figure 1). The fourth cysteine ligand in natural ferredoxins is typically located toward the carboxy terminus with three or more intervening amino acids between it and the core motif.²³² In our initial FdM design this cysteine was placed four positions toward the amino terminus for modular integration into the ferredoxin-heme protein maquette.¹²³a Two peptides derived from alternative natural [4Fe-4S]²⁺/³⁺ binding sequences were designed to test both the flexibility of the nonconsensus motif cysteine placement in the primary structure and the possibility of core motif expansion. The sequence of the first peptide, FdM-Pa shown in Table 1, is derived directly from __P. aerogenes__ containing the identical CIACGAC core motif as FdM but with the remote cysteine, which ligates the second [4Fe-4S]²⁺/³⁺ cluster in the natural ferredoxin, removed three amino acids toward the carboxy terminus. The FdM-Pa and FdM peptides were intended to conserve several of the N—H···S hydrogen bonds local to the [4Fe-4S]²⁺/³⁺ cluster observed in the X-ray structure of __P. aerogenes__ ferredoxin I believed to be involved both in favoring the tetranuclear cluster and modulating its reduction potential.³³ The sequence of the second peptide, FdM-Sp (Table 1), is derived from__Spirulina plantensis__ ferredoxin and contains an expanded CXXXCCXXC core motif with the fourth cysteine removed toward the C-terminus by seven amino acids.³⁴

The prototype FdM and these first two test peptides incorporate [4Fe-4S]²⁺/³⁺ clusters that share nearly identical characteristics of EPR g-values, line shapes and relaxation properties, equilibrium redox potentials, and solution molecular weights, thereby facilitating direct comparison of the [4Fe-4S]²⁺/³⁺ reconstitution yields. The yields were evaluated from their doubly integrated EPR spectra under standard reconstitution conditions relative to a FdM control peptide reconstituted at the same time, as shown in Figure 2 and enumerated in Table 1. They clearly demonstrate that the prototype ferredoxin


maquette (FdM) is significantly more effective at binding and stabilizing the $[\text{4Fe-4S}]^{2+/+}$ cluster over the FdM-Pa (43% relative yield) and FdM-Sp (13%).

Two additional peptides whose sequences were derived from natural $[\text{2Fe-2S}]^{2+/+}$ binding proteins, FdM-SoxR and FdM-Rieske, were synthesized in an initial attempt to construct a $[\text{2Fe-2S}]^{2+/+}$ maquette with cysteine or histidine ligation. The FdM-SoxR peptide, Table 1, was derived directly from the E. coli SoxR protein, which contains a truncated CXC core motif. The primary structure for the 26 amino acid FdM-Rieske was derived from the Rieske iron–sulfur protein of Rhodobacter capsulatus and contains both a CXH and CXXH motif. The primary structure and that these values offer a practical way to assess the relative competency for $[\text{4Fe-4S}]$ incorporation.

**FdM Ligand Removal Variants: Cysteine to Alanine or Leucine.** The effect of replacing a cysteine in FdM designed to ligate the cluster with nonligating residues was evaluated at each of the four cysteine positions. The sequential series of single alanine variants, FdM-1A, FdM-2A, FdM-3A, and FdM-4A (Table 1), was augmented with double Cys→Ala variants, FdM-1,4A and FdM-2,3A, and a single Cys→Leu variant, FdM-3L. Cluster incorporation into these peptides yielded indistinguishable EPR spectral characteristics and equilibrium redox potentials. These iron sulfur peptides differed from one another in terms of their relative yields of reconstitution (Table 1) and peptide:cluster stoichiometry. Replacement of any one of the first three cysteines with alamines (FdM-1A, FdM-2A, and FdM-3A) lowered the observed reconstitution yield to $52\%–56\%$ relative to FdM. A more significant loss of yield, down to $10\%$ of the original FdM, was observed for the FdM-4A peptide, suggesting this cysteine may play a more crucial role in $[\text{4Fe-4S}]$ assembly than the other three. The relative $[\text{4Fe-4S}]$ incorporation yield of the reconstituted FdM-3L peptide (52%) was identical to that of the related FdM-3A peptide, hence, steric bulk at this peptide position appeared to have little to no influence on the incorporation of the iron sulfur cluster. With only three cysteine ligands available in these ferredoxin maquettes with a 1:1 peptide:cluster binding stoichiometry determined by gel permeation chromatography, we assume that the fourth ligand is occupied by an exogenous solvent ($\beta$-ME, water, or hydroxide ion). One of the peptides containing two cysteine and two alanine residues, FdM-2,3A, retained partial competence for $[\text{4Fe-4S}]^{2+/+}$ cluster incorporation (29% relative to FdM), the other, FdM-1,4A, resulted in less than 5% incorporation, further implicating the relative importance of the fourth cysteine in these FdM variants. Gel permeation chromatography demonstrated a 2:1 peptide:cluster stoichiometry.

![Figure 2](image-url)

**Figure 2.** Electron paramagnetic resonance (EPR) characterization of the natural sequence ferredoxin maquettes and ligand variants of FdM. Frozen solution X-band EPR spectra of dithionite reduced FdM, FdM-Pa, FdM-Sp, FdM-SoxR, and FdM-Rieske as well as FdM-3A, FdM-3L, and FdM-1H. EPR spectral acquisition parameters are given in the text. Peptide sequences are given in Table 1.
for FdM-2,3A, indicating the cluster assembled two peptides to satisfy its four ligand coordination sphere requirements.

FdM Ligand Substitution Variants: Cysteine to Aspartate or Histidine. We evaluated whether an oxygen or nitrogen donor could serve as a ligand for the incorporated [4Fe-4S]^{2+/3+} cluster of FdM by independent replacement of the four ligating cysteines with aspartic acid or histidine. All the aspartic acid or histidine ligand variant peptides bound a single [4Fe-4S]^{2+/3+} cluster that had EPR spectral (Figure 2) and redox properties (Figure 3) virtually identical to those of the FdM peptide. Efforts to observe a S = \frac{3}{2} EPR spectral signature, which has been observed for some mutant [4Fe-4S]^{2+/3+} proteins with aspartate ligation,\(^{(38)}\) using various conditions of sample temperature, microwave power, and peptide concentration, resulted only in observation of the \(S = \frac{3}{2}\) EPR signal typical of the FdM variants studied. The lack of spectral changes (g-values, line widths, relaxation properties) and modulation of the redox potential may suggest that these aspartate and histidine residues most likely do not ligate the iron–sulfur clusters; however, there is insufficient evidence in the literature to quantitatively determine the effects of altered cluster ligation on the EPR spectrum or redox potential. However, the lower reconstitution yields viewed in concert with the EPR spectra and redox potentials indicate that these aspartate and histidine residues most likely do not ligate the iron–sulfur clusters.

Discussion

A modular ferredoxin maquette has been utilized to probe the ligand requirements for assembly of a [4Fe-4S]^{2+/3+} cluster under biochemical reconstitution conditions after validation by comparison to other natural sequence ferredoxin maquettes based on [4Fe-4S] and [2Fe-2S] proteins. The systematic replacement of cysteine residues shows that the four cysteine positions are not equivalent in their contribution to cluster yield. In contrast, the nature of the cysteine replacement amino acid (Ala, Leu, Asp, or His) was relatively unimportant in terms of the EPR spectral characteristics and quantity of [4Fe-4S]^{2+/3+} clusters.

FdM fully incorporates a single [4Fe-4S]^{2+/3+} cluster per peptide with redox and spectral characteristics similar to those of the natural [4Fe-4S]^{2+/3+} protein, \(P. aerogenes\) Fd.\(^{(39,40)}\) A lower yield of cluster incorporation is observed for both FdM-Pa, the other ferredoxin maquette based on \(P. aerogenes\) ferredoxin (same core consensus sequence with a C-terminal remote cysteine), and FdM-Sp (with an expanded core motif). The loss of yield in FdM-Pa is somewhat expected since the C-terminal cysteine in this peptide is not positioned to ligate the same cluster as the other three cysteines in the natural protein. The lower yield of FdM-Sp shows that expansion of the spacing between the core motif cysteine ligands is detrimental to the incorporation of the cluster. Although exciting FdM-Pa and FdM-Sp from their native environments may contribute to loss of cluster incorporation, one clear result from FdM is that the secondary and tertiary structure components are not mandatory for the [4Fe-4S] cluster assembly or stability.

Protein structure components may aid in differentiating the nuclearity of the incorporated cluster as illustrated by the incorporation of a [4Fe-4S]^{2+/3+} cluster into the two maquettes derived from natural [2Fe-2S]^{2+/3+} proteins. Clearly, the forces which assemble the [4Fe-4S] cluster can have an overriding effect on these minimal polypeptide ligands just as they do in model complexes where cluster conversion of two [2Fe-2S]^{2+/3+} clusters into a single [4Fe-4S]^{3+} center is observed upon reduction in organic solvents.\(^{(41)}\) Consistent with the thermodynamic stability of the [4Fe-4S] cofactor, ligand deletion mutants of natural [4Fe-4S] proteins often satisfy their metal coordination sphere requirements by recruiting novel amino acid ligands (i.e. ligand swapping).\(^{(42–44)}\) In the case of FdM-Rieske, the assembly of a tetranuclear cluster indicates that the structural disulfide bond juxtaposed to the natural Rieske iron sulfur center\(^{(45–46)}\) may help drive the selective formation of the

---

Footnotes:


Characterization of [4Fe-4S]$^{2+/+}$ Clusters

The FdM variants demonstrate that only three cysteines are required for [4Fe-4S] incorporation into a single peptide (with two cysteines yielding peptide dimerization) and reveal the differential roles each cysteine position plays in cluster incorporation. Replacement of the fourth cysteine, derived from the core CiacGAC motif, with any of the chosen amino acids is highly detrimental to yield compared to the other cysteine positions, illustrating its greater importance in cluster incorporation, whereas the similarity of the reduction in the yield of [4Fe-4S]$^{2+/+}$ cluster bound to FdM-1A, FdM-2A, and FdM-3A demonstrates a similar importance of each of this cysteine trio in cluster assembly.

The replacement of the ligating cysteines with aspartic acid and histidine residues, potentially competent for cluster ligation, illustrates that [4Fe-4S]$^{2+/+}$ proteins with mixed ligation are not readily constructed using this minimal ferredoxin maquette scaffold. The EPR spectral parameters and reduction potentials of these FdM variants were identical to the single cysteine to alanine variant of FdM and their lower cluster incorporation yields were similar, suggesting that the oxygen and nitrogen donor ligands were not binding the cluster. Thus, the mere loss of a cysteine ligand was more important to the yield than the exact identity of the amino acid chosen to replace the cysteine in FdM. While aspartate has been introduced to successfully ligate a [4Fe-4S]$^{2+/+}$ cluster in a site-directed mutant of F$_6$ in Photosystem I, the spectral changes observed in the F$_6$ mutant (EPR line shape of the cluster, $P_{1/2}$ value) were not observable in the FdM variants with aspartate, suggesting that there is insufficient protein matrix available to impose less thermodynamically favorable ligand binding to the cluster, thereby modulating its spectroelectrochemical properties. Consistent with this assertion is the observation that ligand deletion mutants of natural ferredoxins sometimes lose iron to form [3Fe-4S] clusters, recruit nearby cysteines as replacement ligands via ligand swapping, or fail to assemble.

The apparent lack of response of the FdM equilibrium redox potential to the ligand environment (four cysteine or three cysteine and an exogenous ligand) indicates that the high degree of solvent exposure of the [4Fe-4S]$^{2+/+}$ cluster in FdM may limit these local effects on the cluster potential. The high local dielectric may have leveled any charge or dipole effects imposed on the cluster consistent with the observation that surface protein charges do not modulate the reduction potentials. The Coulombic interaction between the cluster and the surrounding protein ($v_{qv}$) has been hypothesized to be a strong controlling factor in iron–sulfur cluster potential determination as implicated by quantitative modeling of iron sulfur protein redox potentials using the Protein Dipoles Langevin Dipoles (PDLD) model. These Coulombic effects are likely to be screened in solvent exposed clusters (dominance of $\Delta V$ by the $v_A$ and $v_B$ terms) such as these 16 amino acid ferredoxin maquettes and $\alpha$-FeS. In contrast to these low-potential designed proteins, the initial designed HiPIP ($E_{1/2} > +300$ mV) resides sufficiently deep within the redesigned hydrophobic core of thioredoxin and might be more responsive to such Coulombic effects although other effects might be dominant.

(49) Scott M. P.; Biggins J. Protein Sci. 1997, 6, 340–346.
The prototype ferredoxin maquette, FdM, successfully mimics the spectroscopic and electrochemical characteristics of a native ferredoxin and provides a robust template upon which to study the ligand requirements for efficient \([4\text{Fe-4S}]^{2+/+}\) assembly in a synthetic peptide using biochemical reconstitution conditions. It is clear from these and previous results\(^{17a}\) that each of the cysteines are important to cluster incorporation and that the cluster dominates these unstructured 16 amino acid peptides providing the standard spectroscopic and electrochemical properties observed for these ferredoxin maquettes. The consistent cluster nuclearity, EPR spectral characteristics, and redox properties of the bound \([4\text{Fe-4S}]^{2+/+}\) clusters clearly illustrate the inherent robustness of the cofactor in these minimal maquettes. Distortions in coordination geometry and/or asymmetric ligation which may be necessary for the modulation of the cluster properties in maquettes are clearly absent when employing minimal peptides without conformational constraints binding to a metal center of considerable thermodynamic stability. Additionally, molecular modeling of metal binding sites toward ideal coordination geometries using rational protein design or computer-aided structure-based redesign of larger protein systems might de facto result in metalloproteins with properties indistinguishable from these maquettes or, in fact, small molecule bioinorganic coordination complexes in isotropic solution.

**Acknowledgment.** This work was supported by the National Institutes of Health (Grant DM41048). B.R.G and F.R. acknowledge receipt of postdoctoral fellowships from the NIH (Grant GM17816) and EMBO, respectively. This work was supported in part by the MRSEC Program of the National Science Foundation under Award Number DMR96-32598. Mass spectroscopic analyses were performed by the Protein Chemistry Laboratory of the University of Pennsylvania. The authors wish to thank Drs. Huangen Ding and Sami Saribas for providing the sequences for design of FdM-SoxR and FdM-Rieske.

**Supporting Information Available:** Figure S1, evaluation of the formation and stability of the FdM \([4\text{Fe-4S}]^{2+/+}\) cluster, and Figure S2, EPR spectral characterization of the single cysteine to aspartic acid variants of FdM at each cysteine position (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA981279A