Formation and characterization of an all-ferrous Rieske cluster and stabilization of the [2Fe-2S]$^0$ core by protonation

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The all-ferrous Rieske cluster, [2Fe-2S]$^0$, has been produced in solution and characterized by protein–film voltammetry and UV-visible, EPR, and Mössbauer spectroscopies. The [2Fe-2S]$^0$ cluster, in the overexpressed soluble domain of the Rieske protein from the bovine cytochrome bc$_1$ complex, is formed at $-0.73$ V at pH 7. Therefore, at pH 7, the [2Fe-2S]$^{1+/0}$ couple is 1.0 V below the [2Fe-2S]$^{2+/1+}$ couple. The two cluster-bound ferrous ions are both high spin ($S = 2$), and they are coupled antiferromagnetically ($J \approx -20$ cm$^{-1}$, $H = -2.5$ G) to give a diamagnetic ($S = 0$) ground state. The ability of the Rieske cluster to exist in three oxidation states (2+, 1+, and 0) without an accompanying coupled reaction, such as a conformational change or protonation, is highly unusual. However, uncoupled reduction to the [2Fe-2S]$^0$ state occurs at pH $> 9.8$ only, and at high pH the intact cluster persists in solution for $< 1$ min. At pH $< 9.8$, the all-ferrous cluster is stabilized significantly by protonation. A combination of experimental data and calculations based on density functional theory suggests strongly that the proton binds to one of the cluster $\mu_2$-sulfides, consistent with observations that reduced [3Fe-4S] clusters are protonated also. The implications for our understanding of coupled reactions at iron–sulfur clusters and of the factors that determine the relative stabilities of their different oxidation states are discussed.

Iron–sulfur (FeS) clusters are essential to all forms of life. Most frequently, they are simple electron carriers, but they also constitute catalytic centers, structural scaffolds, and sensors, and they undergo oxidative degradation (1, 2). The rhombic [2Fe-2S], cuboidal [3Fe-4S], and cubane [4Fe-4S] clusters are the most common, but elaboration of these basic modules has produced clusters that contain heterometals and up to eight iron centers. For example, the catalytic clusters in acyl-CoA synthase contain nickel, and the P-cluster and iron–molybdenum cofactor of nitrogenase can be considered to be two cuboidal subclusters joined by sulfurs. Assembly, disassembly, and interconversion of the simpler clusters are exploited in oxygen sensing and in the control of intracelullar iron levels, and they also constitute enzyme active sites, such as in aconitase, chloroplast ferredoxin:thioredoxin reductase, and biotin synthase. In contrast, uncontrolled cluster disassembly (during oxidative stress) accelerates the production of reactive oxygen species and therefore is potentially very damaging.

Formally, each iron center in a cluster can be ferric or ferrous, so [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters have three, four, and five possible oxidation states, respectively. In proteins, the oxidation states of [3Fe-4S] clusters cover the widest range because many have been observed in the 1+ (all-ferric), 0, and 2− (all-ferrous) states (3). Formation of the [3Fe-4S]$^0$ state is associated with protonation (4), and reduction to the 2− state occurs only upon the uptake of a total of three protons (3). Therefore, the overall charge is conserved, and it is likely that [3Fe-4S] clusters exist in more than two oxidation states because they can be protonated. The protons probably bind on the three $\mu_2$-sulfides, consistent with the ability of [3Fe-4S]$^0$ clusters to coordinate a fourth metal ion (5). In contrast, most [4Fe-4S] clusters in proteins are confined to only two oxidation states, either the 2+ and 1+ states or the 3+ and 2+ states in high-potential iron proteins. The [4Fe-4S] cluster in the Fe-protein of nitrogenase ("the Fe-protein") is unique because it can exist in the all-ferrous state and is stable in more than two oxidation states (2+, 1+, and 0) (6). This versatility may be because of its unusually high solvent accessibility (7), although during turnover it undergoes significant conformational changes coupled to nucleotide binding (8), and protonation of the all-ferrous state has not been excluded. The nitrogenase [8Fe-7S] P-cluster ("the P cluster"), the only high-nuclearity cluster not involved directly in small molecule activation, also exists in three oxidation states, but interconversions between them are coupled to changes in cluster structure and ligand and to protonation; the most reduced state may be all-ferrous (9, 10). There is no confirmed example of an FeS cluster in a protein undergoing sequential uncoupled redox transformations.

Under physiological conditions, [2Fe-2S] clusters have been observed in the 2+ and 1+ states only. The all-ferrous state may not have been observed because the potential for its formation is too negative, the all-ferrous cluster is unstable, or two-electron reduction is precluded in the absence of a charge-compensation mechanism. In contrast, in anaerobic nonaqueous solvents, synthetic [2Fe-2S] analogues can be reduced sequentially by two electrons (11); the reduction potentials are reported to be only $\approx 0.25$ V apart, but the proposed all-ferrous nature of the fully reduced state has not been demonstrated. A [2Fe-2S]$^0$ cluster was generated artificially in spinach ferredoxin by irreversible complexation of the protein to a chromium reductant, increasing the reduction potential significantly and adding extra positive charge (12). Voltammetric signals from the soluble Rieske domain from the bovine heart cytochrome bc$_1$ complex ($-0.84$ V at pH 7, potential reported to be pH independent) were attributed to formation of the all-ferrous cluster, but no characterization was attempted (13). Here, we describe the reversible formation of a stable, unmodified [2Fe-2S]$^0$ cluster-containing protein and its extensive characterization. The [2Fe-2S]$^0$ cluster is a Rieske cluster, so its formation is facilitated by the neutral, electronegative histidine ligands. Our results provide insight into the potentials, stabilities, and reactivities of the different oxidation states of the simplest FeS clusters and are also relevant to understanding the structures and functions of higher nuclearity clusters.

Materials and Methods

Protein Preparation. The design, cloning, overexpression, and purification of the truncated Rieske protein from the bovine cytochrome bc$_1$ complex (BtRp) and its S163A, Y165F, and C144A + C160A (AA) mutants are described in ref. 14. BtRp lacks residues

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Abbreviations: BtRp, overexpressed soluble Rieske protein from the bovine cytochrome bc$_1$ complex; DTPA, diethylenetriamine-N,N,N’,N’’,N’’’-pentaacetate; FeS, iron–sulfur.

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1–69 of the mature protein and has a six-histidine tag on its N terminus. Its spectroscopic properties (UV–visible and EPR) and reduction potential ([2Fe-2S]^{2+/+1}) are very similar to those of its structurally characterized native equivalent, resolved from the complex by proteolysis (14–16).

**Protein-Film Voltammetry.** Protein-film voltammetry was carried out as described in refs. 17 and 18 under anaerobic conditions (O<sub>2</sub> < 2 ppm). Typically, the potential was prepoised at the low-potential limit for 20 s, then scanned at 10 mV s<sup>−1</sup>. Reduction potentials are relative to the standard hydrogen electrode and were independent of scan rate, and poise potential and time.

**Generation of the All-Ferrous Rieske Protein in Solution.** All-ferrous Br<sub>R</sub>p was generated in solution by using Eu<sup>3+</sup>-diethylenetriamine-N<sub>3</sub>N<sub>2</sub>N<sub>2</sub>N<sub>2</sub>-pentaaetate (Eu<sup>3+</sup>-DTPA) (19). All procedures were performed under strictly anaerobic conditions (O<sub>2</sub> < 2 ppm). A stock of 50 mM Eu<sub>2</sub>O<sub>3</sub> (Sigma) in 0.5 M HCl was diluted to 2 mM in 50 mM Hepes/0.3 M NaCl (pH 8.0), final pH was 7, and electrolyzed exhaustively at −0.6 V by using a graphite “pot” electrode (3) to reduce the Eu<sup>3+</sup>. The Eu<sup>3+</sup> concentration was verified spectrophotometrically. The Eu<sup>3+</sup> and protein solutions were brought to the experimental pH and mixed to give a molar excess of Eu<sup>3+</sup> of 1.4–2.0. Then, upon the addition of DTPA to the same molar excess (Sigma, 50 mM DTPA in 0.2 M NaOH), Br<sub>R</sub>p was reduced stoichiometrically to the all-ferrous form.

**Mössbauer Spectroscopy.** Cells expressing Br<sub>R</sub>p were grown on M9 minimal medium, supplemented with 5 mg ml<sup>−1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>–6H<sub>2</sub>O. They were incubated at 37°C until induction at OD<sub>600</sub> was 0.2 with 0.5 mM isopropyl β-D-thiogalactopyranoside and then for 18 h at 25°C. Purified Br<sub>R</sub>p was concentrated from their [2Fe-2S]<sup>0</sup> cluster, pK of the [2Fe-2S]<sup>0</sup> state. The data shown in Fig. 2 reflect a high-potential Rieske proteins from *Rhodobacter sphaeroides* (R<sub>s</sub>R<sub>p</sub>) and *Thermus thermophilus* (T<sub>r</sub>R<sub>p</sub>) are high-potential Rieske proteins from *Rhodobacter sphaeroides* (R<sub>s</sub>R<sub>p</sub>) and *Thermus thermophilus* (T<sub>r</sub>R<sub>p</sub>), respectively. As predicted from their [2Fe-2S]<sup>2+/+1</sup> potentials (18), the [2Fe-2S]<sup>2+/+1</sup> couples in Br<sub>R</sub>p and R<sub>s</sub>R<sub>p</sub> occur at very similar potential (−0.730 and −0.747 V, respectively, at pH 7, but in T<sub>r</sub>R<sub>p</sub> the potential is considerably lower (−0.918 V at pH 7).

**Reduction to the All-Ferrous State Is Accompanied by Protonation.** Protein-film voltammetry measurements showed that the [2Fe-2S]^{2+/+1} reduction potential is strongly pH dependent (Fig. 2). At pH < 9, the gradient of the curve of E vs. pH is −58 mV per decade; therefore, one proton is taken up by the [2Fe-2S]<sup>2+/+1</sup> state. This proton is distinct from the two protons that can be dissociated from the two histidine ligands. In the [2Fe-2S]<sup>2+/+1</sup> state, they are fully bound at pH < 11 (14, 18), and they remain bound at all observable pH values in the [2Fe-2S]<sup>2+/+1</sup> state. The data shown in Fig. 2A are recorded in 2 M NaCl, confirming that the pH dependence is not because of nonspecific changes in protein charge (21); similar behavior was observed in 0.1 and 0.01 M NaCl also. At pH > 10, the reduction potential becomes pH independent (at −0.89 V), defining the pK of the [2Fe-2S]<sup>0</sup> cluster, pK = 9.77, according to the modeled lines shown in Fig. 2, calculated by using Eq. 1 and Fig. 3. The pH-independent region is present unambiguously in D<sub>2</sub>O (Fig. 2B) because the pK decreases to 8.87.

\[
E_{\text{obs}} = E_{\text{alk}} + \frac{RT}{F} \ln \left( 1 + \frac{a_{\text{H}^+}}{K} \right)
\]

**Generation of the All-Ferrous Cluster in Solution.** For spectroscopy, the all-ferrous cluster was produced stoichiometrically by using Eu<sup>3+</sup>-DTPA (E = −1.14 V at pH 8.0) (19). UV-visible and EPR spectroscopies, after reoxidation by [Fe(CN)]<sub>4</sub><sup>−</sup>, and voltammetry demonstrated that formation of the all-ferrous cluster was reversible and confirmed that the properties of the cluster were unaltered. Direct reduction of the [2Fe-2S]<sup>2+/+1</sup>-containing protein by electrolysis in a graphite pot electrode (3) was also possible at −0.9 V (0°C, 20°C).
pH 8) but was marred by rapid reoxidation, even under strictly anaerobic conditions (O$_2$ < 1 ppm). Ti$^{III}$(citrate) and Cr$^{II}$(EDTA) were used previously to reduce the Fe-protein from *Azotobacter vinelandii* (6, 22), but the BtRp [2Fe-2S]$^{1+}/H^+$ cluster was not reduced by Ti$^{III}$(citrate), and the protein precipitated rapidly upon reduction by Cr$^{II}$(EDTA).

Fig. 4 shows the UV–visible spectra of the [2Fe-2S]$^0$ and [2Fe-2S]$^{1+}$ clusters. As expected, the all-ferrous state is pale yellow/green, whereas the more oxidized states are red/brown. Its spectrum is similar to the spectra of the [2Fe-2S]$^0$ state of spinach ferredoxin, generated by complexation to a chromium reductant (12), and the [3Fe-4S]$^{2-}$-3H$^+$ cluster in *Sulfolobus acidocaldarius* ferredoxin (3). Interestingly, the all-ferrous Fe-protein is distinct because it is pink-red, due to an absorption band at $\approx$520 nm (19, 22). The all-ferrous cluster of BtRp is EPR silent, in both perpendicular and parallel mode, indicating that it has zero, or integer, spin.

**Mössbauer Spectroscopy.** Fig. 5A shows the zero-field Mössbauer spectrum of the all-ferrous cluster at pH 7, 4.2 K. Its asymmetric form results from two symmetric quadrupole doublets, with Lorentzian lineshapes and equal intensity (subspectra a and b), and a third doublet with $\approx$16% intensity (subsphere c). The high isomer shifts ($\delta$) and large quadrupole splittings ($\Delta E_Q$) indicate that all three doublets are due to high-spin (S = 2) ferrous iron (23). Subsphere c is caused by adventitious iron because of its high $\delta$ ($>$1.2 mm/s), which exceeds the range for tetrahedral Fe($^{II}$)$S_xX_2$ sites and is typical of six-coordinate Fe$^{2+}$. Subspectra a and b are ascribed to the two tetrahedral cluster iron and demonstrate complete conversion from [2Fe-2S]$^{1+}$ to [2Fe-2S]$^0$. They are clearly distinguishable because of the asymmetric cluster coordination and display remarkably narrow linewidths (\$= 0.25 mm/s, confirming that the sites are highly homogeneous. The absence of magnetic hyperfine splitting is consistent with a diamagnetic (S = 0) or integer spin ground state. Zero-field spectra were recorded also at 160 and 80 K for direct comparison with the paramagnetic [2Fe-
Therefore, the cysteine-coordinated iron (FeC) has tetrahedral FeIIS4 sites in the \([2Fe-2S]_0\) cluster in \(\text{FeII-Cys (n)}\) and \(\text{FeII-His (n)}\); and \(\text{ferrous ion in the \([2Fe-2S]_1\) with positive \(\Delta\Omega\)}\) or perhaps a bridging sulfide is protonated (see below). Analyses of magnetically perturbed spectra (see below) did not agree more closely with those of related proteins and model compounds (24–26). The doublet with higher \(\delta\) (Table 1) is from the histidine-coordinated iron (FeH), because the nitrogen ligands are more electronegative and the Fe–N bond is less covalent (23). Therefore, the cysteine-coordinated iron (FeC) has \(\delta = 0.70 \text{ mm s}^{-1}\) and \(\Delta\Omega = 2.76 \text{ mm s}^{-1}\) at 4.2 K, consistent with values from tetrahedral FeIII4 sites in the \([2Fe-2S]_0\) cluster in \(\text{Aquifex aeolicus}\) ferredoxin (27) (\(\delta = 0.71 \text{ mm s}^{-1}\), \(\Delta\Omega = 2.75 \text{ mm s}^{-1}\) at 4.2 K) and rubredoxin (\(\delta = 0.7 \pm 0.02 \text{ mm s}^{-1}\) at 4.2 K) (28). FeCII has \(\delta = 0.78 \text{ mm s}^{-1}\) and \(\Delta\Omega = 2.24 \text{ mm s}^{-1}\) at 160 K and should match the ferrous ion in the \([2Fe-2S]_1\) clusters of BiRp (\(\delta = 0.69 \text{ mm s}^{-1}\), \(\Delta\Omega = 2.87 \text{ mm s}^{-1}\) at 160 K) and \(\text{and \([2Fe-2S]_2\) clusters in \(\text{FeII-His (n)}\) and \(\text{FeII-Cys (n)}\).\) Increases in \(\delta\) for one cluster iron, upon the reduction of another, have been observed for FeII+ in \([2Fe-2S]^{2+}\) and \([3Fe-4S]^{2+}\) clusters and also for the FeII+ pair in \([4Fe-4S]^{2+}\) clusters (26). For BiRp, the increased \(\delta\) of FeII upon the reduction of FeII+ suggests that reduction weakens the FeII–sulfide bonds. The reason may be that the cluster dimensions increase, hydrogen bonds between cluster and protein strengthen, or perhaps a bridging sulfide is protonated (see below).

Magnetically perturbed Mössbauer spectra (Fig. 5B) were recorded to explore spin coupling in the \([2Fe-2S]_0\) cluster and to establish a lower limit for the spin coupling constant. The magnetic splittings are typical of a diamagnetic system \(S = 0\), demonstrating that the two \(S = 2\) ferrous ions are strongly antiferromagnetically coupled and could be simulated satisfactorily by using the values given in Table 1. No internal fields, arising from the thermal population of excited paramagnetic states, could be detected at up to 80 K (7 T applied field). A quantitative analysis was derived from spin-Hamiltonian simulations by using the usual nuclear Hamiltonian (25) and an explicit exchange coupling term –2\(JS_S^1S_L\). The local axial and rhombic zero-field parameters were \(D_L = 7 \text{ cm}^{-1}\), \(E_D = 0\), and the hyperfine parameters were taken from Table 1, with positive \(\Delta\Omega\). Symmetry parameters \(\eta = 0.8\) (a) and 0.6 (b), and \(\eta = 0.8\) (c), \(\eta = 0.6\) (d), and \(\eta = 0.8\) (e). For the all-ferrous cluster, any simulation with \(-J > 30 \text{ cm}^{-1}\) is in accord with the experimental spectra, consistent with the estimated value from the \([2Fe-2S]_0\) cluster in \(Aquifex aeolicus\) ferredoxin, \(-J > 40 \text{ cm}^{-1}\), derived by using similar conditions (27). Therefore, the \([2Fe-2S]^0\) cluster, like \([2Fe-2S]^1\) and \([2Fe-2S]^2\) clusters, is an antiferromagnetically coupled spin pair. \(J\) is expected to decrease upon reduction because of the weaker coordination of FeII and an increase in the Fe–Fe distance, as observed in, for example, the \([2Fe-2S]^2\) and \([2Fe-2S]^3\) clusters in spinach ferredoxin \((-J = 183 \text{ and } -J > 100 \text{ cm}^{-1}\), respectively) (29). The Rieske \([2Fe-2S]^1\) clusters in \(\text{BiRp}\) and benzene dioxygenase have \(-J = 100\) and 190 cm\(^{-1}\), respectively (30). Therefore, although a meaningful evaluation is precluded without an exact measurement for the \([2Fe-2S]^0\) cluster, the reported value falls within the expected range.

To explore the protonation of the \([2Fe-2S]_0\) cluster, Mössbauer samples were prepared also at high pH (pH > pk). Samples at pH 10, pD 9, and pD 10 all showed considerable cluster deterioration [as evidenced by the high relative intensity (65–75%) of subspectrum c]. Note that very little degradation of the \([2Fe-2S]_0^1\) cluster is observed under equivalent alkaline conditions. Therefore, the unprotonated \([2Fe-2S]^0\) cluster is considerably less stable than its protonated counterpart. This finding is consistent with the lack of voltammetric signals at high pH (in contrast, the \([2Fe-2S]^{2+}\) couple can be observed even at pH 14 (18)). Similar behavior was reported for the \([3Fe-4S]^2\) cluster (3). The high-pH spectra (Fig. 6) could be fitted by using essentially the same subspectra as the low-pH spectra (Fig. 5A), with equal intensities for subspectra a and b, and values are reported in Table 1. The \(\delta\) values at high pH are lower than at low pH. The protonation of the \([2Fe-2S]^1\) cluster and its protonation sensitivity are consistent with the expected change in the iron monodentate amine coordination, as suggested by the X-ray crystallography (4).
within 0.01 cm⁻¹ of their corresponding low pH values, but all of the 
ΔE₀ values have increased, suggesting small differences in the 
coordination symmetry (23). The increased linewidths probably 
reflect the mixture of states present at pH ≈ pK. No differences in
δ or ΔE₀ were observed between protonated and deprotonated
states of the [3Fe-4S]⁺ cluster of Azotobacter vinelandii ferredoxin I,
where the proton is thought to bind to a μ₂-sulfide (31).

The Site of Protonation. The reduction potential of the AA mutant
is pH dependent at all observable values (pH 4.5–9, −58 mV per
decade), and at pH 7 the reduction potential is equal to that of WT
BtRp (−0.729 V vs. −0.730 V). Therefore, the proton does not bind
to the disulfide. The behavior of mutants S163A and Y165F,
however, is distinct from that of WT BtRp. S163 hydrogen bonds
to the cluster μ₂-sulfide S1, and Y165 hydrogen bonds to S₀ of
the cluster ligand C139 (15). S163A and Y165F both have pH-
dependent reduction potentials (−58 mV per decade) and at pH 7,
where the [2Fe-2S]₀ state is protonated, their reduction potentials
are close to that of WT BtRp, −0.730 V and −0.770 V, respectively.
However, the pK values of the [2Fe-2S]₀ states are altered signif-
ically, to outside the experimental range (pK ≈ 11, EₚK ≈ −1.0 V).
This finding is consistent with the electrostatic influence of the
hydrogen-bonding dipole, which increases the electron affinity of
the cluster and decreases its proton affinity. At pH 7, reduction is
electronneutral, and the two effects cancel. Importantly, this ob-
servation suggests strongly that the sites for reduction and protonation
are spatially close together and inaccessible to solvent, indicating
that the proton-binding site is on (or closely associated with) the
cluster. Similar behavior was described in ref. 32 for the [3Fe-4S]⁺ cluster in
Azotobacter vinelandii ferredoxin I. The behavior of the
BtRp [2Fe-2S]⁺+⁺ couple is strikingly different, because cluster
reduction is coupled to deprotonation of the solvent-accessible and
distant histidine ligands, so the S163A and Y165F mutations affect
reduction potential much more than they affect pK (14).

Therefore, possible protonation sites are the cluster μ₂-sulfides,
as proposed for [3Fe-4S]²⁺ and [3Fe-4S]³⁺ clusters (3, 4), and the S₀
groups of the two cysteine ligands. Coordination of a ferrous heme
iron by neutral cysteine has recently been demonstrated (33).
Calculations of the protonation probabilities of all other titratable
residues in the [2Fe-2S]⁺⁺ cluster and [2Fe-2S]₀ clusters (assuming no
significant structural changes) revealed no significant dependence
on redox state for any residue (20). This finding supports the
proposal that the proton binds to the cluster core. Density func-
tional theory calculations indicated that the geometries of the
unprotonated [2Fe-2S]₀ cluster, and each protonated cluster, were
very similar to the geometry of the [2Fe-2S]⁺⁺ cluster (15).

Comparison of the calculated macroscopic pK values for each
protonation site with the experimental results (Table 2) indicates
that the proton binds to a cluster μ₂-sulfide, not to a cysteine
S₀. The calculated value for WT BtRp differs from the experimental
value by only 1 pH unit, and, consistent with observed trends,
calculated pK values for S163A and Y165F are higher. Consider-
ation of the pK values in S163A and Y165F (to rule out the
influence of the side-chain hydrogen bonds) shows that, as ex-
pected, the two sulfides and two cysteine S₀s are differentiated by
the local environment. In WT BtRp, the side-chain hydrogen bonds
from S163 and Y165 strongly disfavor protonation on S1 and C139,
respectively, so calculations were performed for both the native
conformation and the conformations with the hydrogen bonds
broken. The free energy costs of breaking the hydrogen bonds were
calculated to be equivalent to 2.1 (S163) and 5.6 (Y165) pK units
(in the deprotonated state). Thus, in the absence of the hydrogen
bond from S163, the free energy for protonation of S1 corresponds
to a pK of 10.9, close to the value in S163A. Interestingly, some
of the energy required to break the hydrogen bonds is offset by the
formation of new interactions between the protons bound at S1 and
C139 and the O²⁻ atoms of S163 and Y165, respectively. The net
result of the local environment and the hydrogen bonding is that the
protonation of S1 is favored slightly over the protonation of S2.
However, this difference is small compared with the clear prefer-
ence for protonation on one of the sulfides, rather than on one of
the cysteine ligands, which is maintained consistently.

Table 2. Calculated microscopic pK values for the addition of a single proton to the
μ₂-sulfides of the cluster and S₀ of the cysteine ligands and comparison of calculated
macroscopic pK values with the experimental results

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cysteine ligands</th>
<th>μ₂-sulfides</th>
<th>Macroscopic pK values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C139</td>
<td>C158</td>
<td>S1</td>
</tr>
<tr>
<td>WT</td>
<td>2.1</td>
<td>0.5</td>
<td>8.8</td>
</tr>
<tr>
<td>S163A</td>
<td>2.0</td>
<td>1.3</td>
<td>10.5</td>
</tr>
<tr>
<td>Y165F</td>
<td>4.1</td>
<td>1.2</td>
<td>9.3</td>
</tr>
</tbody>
</table>

Discussion

The Reduction Potential of the [2Fe-2S]⁺⁺ Couple. In BtRp, the
[2Fe-2S]⁺⁺ reduction potential is −0.73 V at pH 7, very close to the
[3Fe-4S]⁰²⁺ potential, typically −0.70 V at pH 7 (3). It is also
comparable to the lowest reported [4Fe-4S]⁺⁺⁺ potential (−0.72
V) (34) and to the potentials of cluster F₆ in type I reaction centers
(approximately −0.70 V) (35) and the [4Fe-4S]⁺⁺⁺ couple in the
Fe-protein of nitrogenase (−0.79 V at pH 8) (6). However, the
neutral and electron-negative histidine ligands, the hydrogen bonds,
and the control of solvent accessibility all combine to raise the
[2Fe-2S]⁺⁺⁺ potentials of high-potential Rieske clusters (18), and
therefore they influence the [2Fe-2S]⁺⁺⁺ potential also. In an
all-cysteine-ligated cluster, the [2Fe-2S]⁺⁺⁺ potential is likely to be
significantly lower. In BtRp, the pH-independent 2+/1+ and 1+/0
potentials (histidines always neutral, cluster always deprotonated)
are separated by 1.2 V. In all-cysteine-ligated clusters the 2+/1+
couple occurs typically at −0.2 V, giving an estimated value of −1.4
V for the 1+/0 couple.

The BtRp cluster is unique, so far, in being able to adopt three
oxidation states without using a coupled protonation or conforma-
tional change to compensate for the extra charge (see below).
Therefore, it is not surprising that the separation between the two
redox couples is so large. For the [3Fe-4S] cluster in S. acidocal-
darius ferredoxin, the 1+/0 and 0/2− couples are separated by only
0.45 V at pH 7 (3), but the separation varies with pHi, and the pK
values of the 2− state are not known. Therefore, the free energy
of protonation cannot be deconvoluted from that of electron transfer.
In the Fe-protein, the most recent value for the [4Fe-4S]⁺⁺⁺ potential
(−0.79 V at pH 8) is only 0.48 V away from the 2+/1+ potential (6), but the Fe-protein is known to undergo significant
conformational changes (8). The high-potential iron protein ([4Fe-
4S]⁺⁺⁺) of Rhodopila globiformis can be reduced to the [4Fe-4S]⁺⁺⁺
state, but the 2+/1+ potential is ill-defined because equilibrium is
never attained, and reduction seems gated by an undefined con-
formational change (36). Finally, in the [8Fe-7S] P-cluster, the

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$P^{2+/3+}$ and $P^{3+/4+}$ potentials actually cross at pH $\sim 7.4$ (10), but the cluster undergoes large redox-state-dependent structural changes (9), as well as protonation. Therefore, in FeS clusters, adjacent redox couples appear to become closer as the cluster nuclearity increases because of the more sophisticated methods of charge compensation that are adopted. Finally, note that the aquo-ions of first-row transition metals, such as Cr and Mn, exist in a wide range of formal oxidation states but that despite the polarity and high dielectric of water, no more than two oxidation states ever adopt the same coordination environment. Consequently, the 0.25-V separation between the proposed $2+/1+$ and $1+/0+$ couples in model [2Fe-2S] compounds (11) is likely to include an unidentified coupled reaction (such as protonation or ion pairing) or to be because of ligand, not iron, reduction.

The Stability of the [2Fe-2S] Cluster. Despite significant contemporary interest, the factors that influence the stability of FeS clusters in their various oxidation states, and the reasons they degrade during oxidative stress, are not well understood. One may question the existence of all-ferrous clusters on the basis of Fe$^{2+}$ being largely in the Irving–Williams series. The Irving–Williams series suggests that the Fe$^{2+}$/S$^{2-}$ bonds in [2Fe-2S]$^{2-}$ are weaker than the Fe$^{3+}$/S$^{2-}$ bonds in [2Fe-2S]$^{3-}$, consistent with the weaker antiferromagnetic coupling. However, in aqueous solution, the relative stabilities are determined by the difference in free energy between [2Fe-2S]$^{2-}$ and $2[Fe(H_2O)]^{3+}$, and [2Fe-2S]$^{2-}$ and $2[Fe(H_2O)]^{3+}$. Fe$^{3+}$ is a "hard" acid, with a preference for O rather than S ligands, but Fe$^{2+}$ is "softer" and more compatible with S. Therefore, all-ferrous clusters may be even less susceptible to degradation than all-ferrous clusters. Indeed, stable all-ferrous [3Fe-4S], [4Fe-4S], [7Fe-8S] clusters have been reported, and the highly oxidized clusters. Indeed, stable all-ferrous [3Fe-4S], [4Fe-4S], [7Fe-8S] (P), of positive charge as Fe$^{II}$ (38).

In BtRp, the unprotonated [2Fe-2S]$^{0}$ cluster does not persist in solution, but it is stabilized significantly by protonation, probably on one of the $\mu_2$-sulfides. It is unlikely that bonding interactions within the [2Fe-2S]$^{0}$ core are strengthened by protonation (for example, antiferromagnetic coupling in [2Fe-2S(R)]) is weaker than in [2Fe-2S]) (25). Instead, it is likely that the surrounding protein structure is destabilized by the addition of two electrons to the cluster, without charge compensation, but that two electrons and a proton can be accommodated. For example, the hydrogen-bonding network may be disrupted, the orientation of backbone dipoles altered, or the solvent structure changed. Note that [4Fe-4S]$^{2+/3+}$ clusters disassemble when oxidized to the 3+ (or 4+) level but that a stable [3Fe-4S]$^{3+}$ cluster of equal charge may be formed also, by expulsion of positive charge as Fe$^{III}$ (38).

Protonation of the [2Fe-2S]$^{0}$ Cluster. The evidence presented here suggests strongly that the [2Fe-2S]$^{0}$ cluster is protonated on one of its $\mu_2$-sulfides, not on a cysteine S. Therefore, the $\mu_2$-sulfides of [2Fe-2S] and [3Fe-4S] clusters distinguish them from [4Fe-4S] clusters, which contain only $\mu_3$-sulfides, by allowing them to compensate for reduction by direct protonation of the cluster core. Cluster basicity increases upon reduction, so that [3Fe-4S]$^{3+}$ clusters are not protonated, but one and three protons are bound in the 0 and 2− states, respectively (3, 4); the basicity of the [3Fe-4S]$^{0}$ core may be enhanced by the tridentate "crown" of sulfide ligands. In [2Fe-2S] clusters, the sulfide lone pairs point away from one another, so the cluster must be reduced to the all-ferrous state to exhibit a quantifiable proton affinity.

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