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Proton Coupling to $[4Fe\text{-}4S]^{2+/1+}$ and $[4Fe\text{-}4Se]^{2+/1+}$ Oxidation and Reduction in a Designed Protein

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Iron−sulfur (Fe−S) center modules are ubiquitous in biochemistry, serving diverse roles ranging from simple biological electron transfer to chemical catalysis.[1] Metal sites in natural proteins are becoming increasingly recognized as fundamental mechanical units involved in biological proton pumping since metal cofactor oxidation/reduction can be accompanied by proton release/uptake as observed in the natural Fe−S proteins NADH−quinone oxidoreductase at cluster N2, the Reiske iron−sulfur protein, the P-cluster of nitrogenase, and *Azotobacter vinelandii* ferredoxin I, *Av*FdI.[2]

We are utilizing peptide-based $[4Fe\text{-}4S]^{2+/+}$ coordination complexes, the ferredoxin maquettes, as aqueous soluble and stable synthetic analogues of natural ferredoxins.[3] The minimal size of the prototype ferredoxin maquette and its spectroscopic and electrochemical resemblance to natural [4Fe-4S] proteins make it a useful model system to probe the fundamentals of [4Fe-4S] protein engineering. To date, the ferredoxin maquettes have provided insight into $[4Fe\text{-}4S]^{2+/+}$ protein sequence design by delineating the role of ligand[3b] and nonligand[3c] amino acid residues in cluster stability.

We show herein that a novel ferredoxin maquette, **IGA**−[4Fe-4S], displays a pH-dependent equilibrium midpoint reduction potential. The data are consistent with a proton-coupled electron-transfer event akin to those observed in natural proteins involved in biochemical proton pumping. The design of **IGA** (*NH_2*-KLCEGG·C**IG**CG**A**C·GGW-*$CONH_2$*) is based on the consensus [4Fe-4S] binding motif of Clostridial ferredoxins[4] ·C**IG**CG**A**C· and is related to the prototype ferredoxin maquette by a single-residue change at position 9 (Ala→Gly).[5]

The spectroscopic properties of this [4Fe-4S] protein maquette are identical to those of the prototype ferredoxin maquette and reminiscent of natural $[4Fe\text{-}4S]^{2+/+}$ proteins. Optical spectroscopy of the oxidized **IGA**−$[4Fe\text{-}4S]^{2+}$ complex (λ_{max} at 310 and 385 nm; ϵ of 23 200 M^{-1} cm^{-1} and 16 100 M^{-1} cm^{-1}, respectively) is fully consistent with the S→Fe(III) LMCT bands observed in natural[6] and designed[7] $[4Fe\text{-}4S]^{2+}$ proteins. Reduction by sodium dithionite results in a bleaching of the UV−vis spectrum and an axial EPR spectrum with *g*-values (2.05, 1.93, and 1.89) indicative of a low potential $S = {}^1/_2$ $[4Fe\text{-}4S]^{+}$ cluster. Thus, the oxidized and reduced state spectral properties of this maquette are indistinguishable from the prototype ferredoxin maquette as well as the designed α_4-FeS[7a] of Scott and Biggins.

The electrochemistry of the **IGA**−$[4Fe\text{-}4S]^{2+/+}$ complex was assayed at 25 °C using redox potentiometry monitored by UV−visible spectroscopy as shown in Figure 1. The observed decrease in the LMCT absorption at 385 nm upon lowering the solution potential is accurately described by a single $N = 1$ Nernst equation. The equilibrium midpoint reduction potential at pH 7.5, $E_{m7.5}$, of

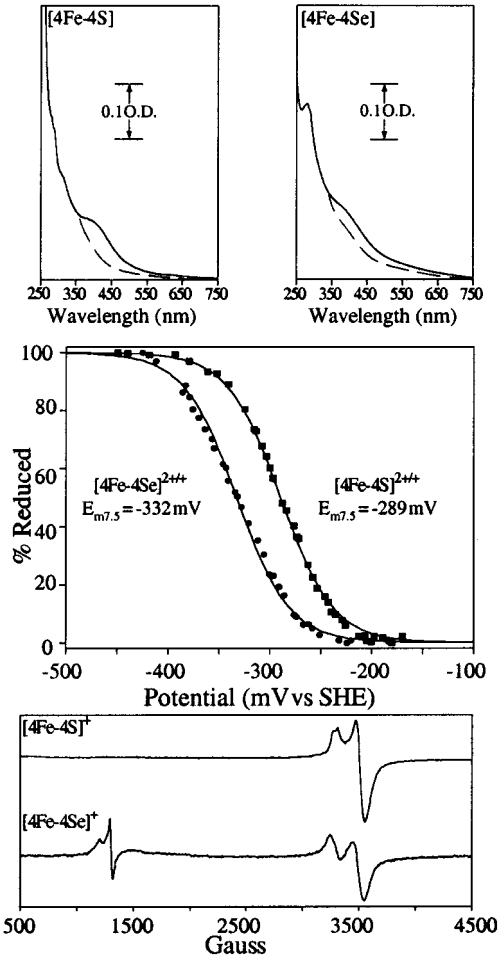

Figure 1. Spectroscopic characterization of **IGA**−$[4Fe\text{-}4S]^{2+/+}$ and **IGA**−$[4Fe\text{-}4Se]^{2+/+}$. (Top) UV−visible spectra of **IGA**−[4Fe-4S] and **IGA**−[4Fe-4Se] in the oxidized (solid line) and reduced states (dashed line). (Middle) Potentiometric titration of **IGA**−[4Fe-4S] and **IGA**−[4Fe-4Se] at pH 7.5. (Bottom) X-band EPR spectra of reduced state **IGA**−$[4Fe\text{-}4S]^{+}$ and **IGA**−$[4Fe\text{-}4Se]^{+}$ recorded at 10 K, 1 mW microwave power, 8 G modulation amplitude.

−289 ± 6 mV vs SHE is consistent with values observed for natural [4Fe-4S] ferredoxins. The redox activity of **IGA**−$[4Fe\text{-}4S]^{2+/+}$ is slightly more positive than both the prototype ferredoxin maquette and α_4-FeS, −350 ± 15 mV (pH 8) and −422 (pH 8.3) mV, respectively. Thus, the amino acid sequence change Ala→Gly has an effect on the cluster electrochemistry.

The reduction potential of the **IGA**−$[4Fe\text{-}4S]^{2+/+}$ complex displays a pH dependence between pH 7 and 11 as shown in Figure 2. The observed redox-Bohr effect illustrates a 60 mV/pH unit slope between the oxidized and reduced pK_a values, pK_a^{ox} and pK_a^{red},

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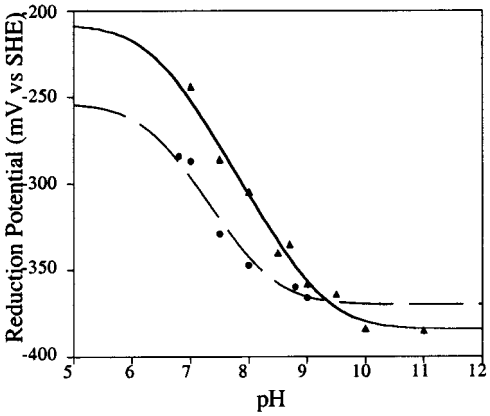

Figure 2. pH dependence of the reduction potential of **IGA**−[4Fe-4S]$^{2+/+}$ (triangles, solid line) and [4Fe-4Se]$^{2+/+}$ (circles, dashed line)

demonstrating a one proton per electron-coupled event. A pK_a^{red} value of 9.3 ± 0.1 is measured from a fit to the redox data. The redox data alone cannot determine the oxidized pK_a^{ox} precisely, due to [4Fe-4S]$^{2+}$ cluster decomposition below pH 7.0, however, an upper limit of pK_a^{ox} ≤ 6.5 can be placed.

The proton concentration required to dissociate the cluster from the peptide was investigated to place a lower limit on pK_a^{ox}. As the pH is decreased below pH 7.5, the UV−visible spectrum of **IGA**−[4Fe-4S]$^{2+}$ bleaches consistent with cluster decomposition. The data are accurately described by a single protonation event with a pK_a value of 6.35 ± 0.05. Thus, cluster stability places a lower practical limit on pK_a^{ox} at 6.35. Using this pK_a^{ox} value yields a theoretical E_{m5} value of −209 mV vs SHE. Thus, proton coupling can perhaps evince a 175 mV (4.1 kcal/mol) effect on the cluster reduction potential (130 mV observed in Figure 2), demonstrating the significant role protons can play in adjusting [4Fe-4S] cluster E_m values.[8]

In an initial attempt to locate the site of proton binding upon reduction of the [4Fe-4S] cluster, we have synthesized the [4Fe-4Se]$^{2+/+}$ cluster in **IGA** using modified literature procedures.[9] After dialysis to remove excess iron, selenide, and dithiothreitol, the incorporation of a single [4Fe-4Se]$^{2+}$ cluster into the **IGA** peptide was confirmed chemically (Fe analysis) and spectroscopically (UV−vis and EPR shown in Figure 1).

The spectroscopic properties of this [4Fe-4Se]$^{2+/+}$ protein maquette are strikingly similar to those of [4Fe-4Se]$^{2+/+}$ substituted *Clostridium pasteurianium* ferredoxin, *Cp*Fd.[10] Figure 1 shows the UV−vis spectrum of the oxidized **IGA**−[4Fe-4Se]$^{2+}$ complex, λ_{max} at 290 and 386 nm; ϵ of 42 600 M^{-1} cm^{-1} and 22 600 M^{-1} cm^{-1}, respectively. One electron reduction of **IGA**−[4Fe-4Se]$^{2+}$ by sodium dithionite yields a complex EPR spectrum with *g*-values indicative of a mixture of $S = {}^1/_2$ ($g = 1.90$, 1.96, and 2.07), $^3/_2$ ($g \approx 4.5$) and $^7/_2$ ($g = 5.17$ and 5.63) spin-states. The EPR spectrum of **IGA**−[4Fe-4Se]$^{+}$ is nearly identical to that observed for *Cp*Fd[10] and distinct from small-molecule complexes such as [4Fe-4Se]-$(SR)_4$.[11]

The equilibrium midpoint reduction potential of **IGA**−[4Fe-4Se]$^{2+/+}$ is −332 ± 6 mV at pH 7.5, a value slightly lower than the corresponding [4Fe-4S] cluster. Figure 2 shows that the reduction potential of the **IGA**−[4Fe-4Se]$^{2+}$ complex is pH dependent over the range of pH 6.8 to 9.[12] The Pourbaix diagram shows a 60 mV per pH unit slope with the pK_a^{red} value of 8.3 ± 0.2, suggesting that the bridging sulfides of the cluster are involved in modulating proton uptake. This indicates a μ_3-sulfide or Cys ligand protonation site. The pH titration of **IGA**−[4Fe-4Se]$^{2+}$ indicates a pK_a^{ox} value of ≤ 6.29. These results are consistent with the conclusions from studies of both small molecule [4Fe-4X](YR)$_4$ (X,Y= S or Se) complexes in aqueous and micellar solutions[13] and the recent detailed study of *Av*FdI, a [3Fe-4S] protein.[2f] Thus, the more negative reduction potential of **IGA**−[4Fe-4Se]$^{2+/+}$ as compared to **IGA**−[4Fe-4S]$^{2+/+}$ at pH 7.5 is a consequence of the alteration in the reduced state pK_a^{red} value; their values are coincidentally identical at pH 9.2.

In the present work, we have demonstrated a proton-coupled electron-transfer event in a peptide-based [4Fe-4S] synthetic analogue. Furthermore, we have illustrated the significant effect protons can have on cofactor equilibrium midpoint reduction potentials in simple peptide−cofactor coordination complexes. Our results indicate protonation of iron ligands must be considered in [4Fe-4S] redox-Bohr effects. Our efforts are now focused on precise determination of the site of proton coupling in this and related [4Fe-4S] maquettes as well as delineating the factors influencing the relevant pK_a values.

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