Backbone-Engineered High-Potential Iron Proteins: Effects of Active-Site Hydrogen Bonding on Reduction Potential

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The factors governing the electron-transfer properties of nonheme iron proteins have been the subject of extensive mutagenesis,[1−5] theoretical,[6−8] and small-molecule modeling studies.[9−12] Structural studies have revealed that highly conserved networks of hydrogen bonds between backbone amides and metal-bound ligands are common features of metalloprotein active sites.[13−16] While these interactions likely serve a structural purpose, backbone amide-ligand interactions are also thought to play a functional role in the regulation of metalloprotein reduction potentials, stabilizing lower metal oxidation states (i.e., the reduced form of the protein).[17−19] However, owing to the difficulty of removing backbone active-site contacts without altering the overall protein fold, a direct experimental assessment of their effect on metalloprotein redox potentials has remained elusive.

Proteins with tetranuclear iron−sulfur cofactors such as ferredoxins (Fds) and high-potential iron proteins (HiPIPs) typically function as electron-transfer proteins.[4,20] While these two families of proteins have Fe_4S_4 cluster active sites with nearly identical metrical parameters,[16] Fds cycle between $[Fe_4S_4]^{1+}$ and $[Fe_4S_4]^{2+}$ states with potentials between −400 and −600 mV (vs NHE), while HiPIPs utilize $[Fe_4S_4]^{2+}$ and $[Fe_4S_4]^{3+}$ cluster oxidation states operating at significantly higher reduction potentials, from ∼+ 200 to +400 mV. Comparison of Fd and HiPIP protein structures reveals a greater number of conserved active-site hydrogen bonds in Fds (8) than in HiPIPs (5), and this difference has been proposed to contribute to the striking difference in redox thermodynamics.[17,21,22]

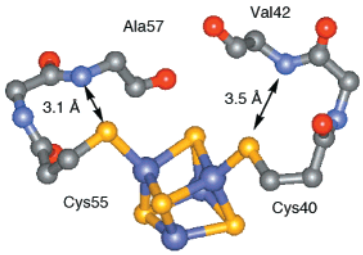

Figure 1. Detail of the HiPIP active-site environment. The Fe_4S_4 cluster and residues 40−42 and 55−57 are shown as ball-and-stick figures. The side chains of noncoordinating amino acids are omitted for clarity. Amides replaced with esters are shown with the corresponding distance from amide nitrogen to sulfur: Val42 N → Cys40 S, 3.5 Å. Ala57 N → Cys55 S, 3.1 Å. The illustration was created using WebLab Viewer (Molecular Simulations, Inc.) with coordinates obtained from PDB file 1ISU.

If NH···S hydrogen bonds stabilize the reduced electronic states of the Fe_4S_4 cluster, removing these interactions should lead to proteins with lower reduction potentials.[17,22] We have elected to probe the functional effects of backbone NH···S hydrogen bonding on metalloprotein active sites by chemically synthesizing a family of proteins based on the *Rhodocyclus tenuis* HiPIP sequence.[20] The redox thermodynamics of these proteins are essential to their function as electron donors to photosynthetic reaction centers.[23−25] The crystal structure of the HiPIP from *R. tenuis* reveals five conserved H bonds involving active-site sulfur-backbone amide interactions.[26] The shortest hydrogen bond involves the backbone amide of Ala57 and the metal-bound sulfur of Cys55 (3.1 Å). A somewhat longer distance separates the backbone amide nitrogen of Val42 and the sulfur of Cys40 (3.5 Å) (Figure 1). In addition to the wild-type protein, we have synthesized two backbone-engineered analogues, [O]Val42 HiPIP and [O]Ala57 HiPIP, in which specific H bonds have been removed by placing ester linkages at positions 42 and 57, respectively.[27]

Chemical protein synthesis is a powerful tool for the study of the molecular basis of protein function[28,29] that allows the straightforward preparation of protein molecules with non-native backbones.[30−32] For each of the three proteins, a native chemical liga-

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tion at cysteine was used to join unprotected synthetic peptides in aqueous solution to give full-length apoprotein.[33] Each analogue was made by joining the N-terminal peptide and one of three C-terminal peptides with the appropriate backbone composition. The N-terminal segment HiPIP (1−24) was prepared, using optimized Boc solid-phase peptide synthesis (SPPS) on thioester-generating resin, and was a synthetic precursor to all three proteins.[34] Backbone-engineered variants of the C-terminal peptides [O]Val42 HiPIP (25−62) and [O]Ala57 HiPIP (25−62) were prepared by incorporating the appropriate hydroxy acid into the synthesis; (L)-lactic acid was substituted for Ala57 in [O]Ala57 HiPIP (25−62), and (*S*)-hydroxymethylbutyric acid was used in place of the native Val42 in [O]Val42 HiPIP (25−62). The appropriate hydroxy acid was preactivated with diisopropylcarbodiimide (DIC) and hydroxybenzotriazole and allowed to react with the deprotected amino terminus of the peptide chain at room temperature for 1 h. Following a DMF flow wash, the appropriate amino acid was then activated with DIC and coupled to the hydroxy group. The reactions were allowed to proceed for either 1 h ([O]Ala57) or overnight ([O]Val42), after which the peptides were completed with standard Boc SPPS.

Following cleavage from the resin by treatment with anhydrous HF and purification by HPLC, the peptide segments were joined by a native chemical ligation reaction. The appropriate HiPIP (25−62) peptide was dissolved with the HiPIP (1−24) α-COSR in 6 M guanidine hydrochloride, pH 7. The reactions were monitored by analytical HPLC and electrospray mass spectrometry (ES-MS). Ligation reactions were complete after stirring overnight in the absence of exogenous thiol catalysts.[35] The full-length polypeptides were then isolated by preparative HPLC and assayed for purity by ES-MS. Fractions containing the desired product were pooled and lyophilized.

Insertion of the Fe_4S_4 cluster was accomplished by treating a solution of apoprotein with a 4-fold excess of ferrous sulfate and sodium sulfide under a nitrogen atmosphere. The resulting dark-brown solution was then desalted by gel filtration. Electrospray mass spectra of the colored eluant revealed the presence of holoprotein with some apoprotein and related degradation products. The holoproteins were purified by ion-exchange FPLC. Colored fractions were analyzed by ES-MS, and fractions containing the holoprotein were collected and pooled. Experimentally determined molecular masses were in excellent agreement with calculated values; WT HiPIP, calcd (average isotope composition): 6645.9 obsd: 6646.4 ± 1.5 amu, [O]Val42: calcd 6646.9, obsd, 6646.6 ± 0.8 amu, [O]Ala57, calcd, 6646.9, obsd: 6647.3 ± 0.8 amu.

All of the HiPIP analogues were brownish-green in their resting state due to broad ligand to metal charge transfer (LMCT) transitions in their UV−vis spectra. The wild-type and [O]Val42 analogue had indistinguishable UV−vis spectra, $\lambda_{max} = 250$, 355 nm, in excellent agreement with values reported for recombinant protein.[36] The low-energy LMCT manifold of the [O]Ala57 analogue was broadened slightly, but the spectrum retains similar λ_{max} values at 250 and 355 nm. The circular dichroism spectra of the three proteins were also similar, each featuring a minimum at 204 and an inflection at 225 nm, indicating that the overall secondary structure of the proteins was not significantly perturbed by the replacement of the amide bond with an ester.

The reduction potentials of WT, [O]Ala57, and [O]Val42 HiPIP were determined by direct electrochemical measurements using an unmodified edge-plane graphite working electrode.[37] Square-wave (Figure 2) and cyclic voltammograms (Figure S5) obtained for the three HiPIP analogues all exhibited reversible electrochemistry in buffered aqueous solution (10 mM ammonium acetate, pH 6).[37] The synthetic wild-type protein was found to have an $E°_{1/2}$ of 326 ± 10 mV vs NHE, typical for a high-potential iron protein.[38] The potentials of the analogues were shifted to significantly lower values: [O]Ala57 (200 ± 10 mV) and [O]Val42 (240 ± 10 mV). These findings are consistent with the proposal that an active-site H bond stabilizes the reduced form of the protein by attenuating charge density on the metal-bound S.[14,18]

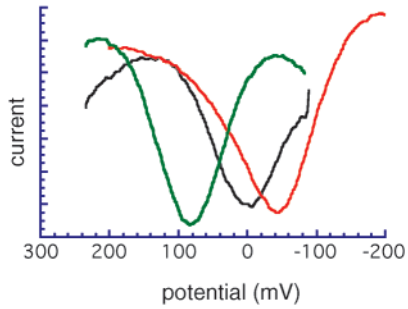

Figure 2. Square-wave voltammograms of WT HiPIP (green line), [O]-Val42 HiPIP (black line) and [O]Ala57 HiPIP (red line). Data are shown as collected vs SCE. Observed potentials: wild-type, + 326; [O]Val42, + 240; [O]Ala57, + 200 ± 10 mV vs NHE.

Chemical synthesis has provided access to a family of metalloproteins in which the effects of active-site hydrogen bonding on redox potentials may be directly measured. Removal of single key active-site hydrogen bonds results in significantly lowered reduction potentials. Our results provide a direct experimental test of predictions based on crystallographic,[15,17,26] spectroscopic,[22,39] small molecule,[11,40−42] mutagenesis,[3,43] and theoretical studies[18,44,45] that predict a functional role for active-site amide NH···S hydrogen bonding and its associated electrostatic interactions. By decreasing charge density on the ligand sulfur atoms, these interactions favor lower metal oxidation states and more positive reduction potentials. Since this mode of hydrogen bonding is a motif found in many metalloproteins, modification of these interactions by backbone engineering may prove to be a useful method for controlling redox properties in a rational manner.

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Supporting Information Available: Detailed experimental procedures, representative HPLC chromatograms and cyclic voltammograms, electrospray mass, UV−vis absorption, and circular dichroism spectra (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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