Influence of Oxygen Ligation on [Fe_4S_4] Cluster Properties. Characterization of the Cys77Ser Mutant of *Chromatium vinosum* HiPIP

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Received October 13, 1995

Chromatium vinosum high-potential iron protein (HiPIP) is a low molecular weight ($M_r \approx 9600$) [Fe_4S_4] cluster protein for which we have designed a high-yielding overexpression system.[1] By systematic mutation of residues in the active site, we hope to evaluate their functional role in maintaining the stability and redox characteristics of the prosthetic center. Previously we have identified a crucial role for Tyr19 in the exclusion of solvent water from the cluster binding pocket.[2] Solvent accessibility results in kinetically facile oxidation and hydrolytic instability. In this paper, we focus on the cluster binding residue Cys77, and discuss the influence of systematic mutations at this site on the stability, redox, and magnetic properties of the iron−sulfur center. Serine mutation of cluster-bound Cys has previously yielded a semistable [Fe_2S_2] cluster in a low-potential ferredoxin.[3] Here we describe an example of a stable, isolable, serine-ligated iron−sulfur center in a high-potential iron protein.

Four mutants were designed (Cys77Ser, -Ala, -Asp, and -Tyr), and expression was attempted in *E. coli*;[4] however, only Cys77Ser demonstrated sufficient stability for isolation as the holoprotein. This was obtained according to standard protocols,[1] and purity was confirmed by SDS/PAGE. The expression yield (∼5 mg/L) was considerably lower than that for recombinant native protein (∼30 mg/L). The protein was stable in the reduced state but was found to degrade within a few hours in the oxidized state. This was accompanied by reduction of residual holoprotein, the decomposed cluster presumably providing the reducing equivalents. The reduction potential and thermodynamic parameters of the Cys77Ser mutant were determined by standard spectrochemical titration and variable temperature methods,[7] using a ferro-/ferricyanide couple to poise the solution potential and serve as a mediator. The solution absorbance was monitored at 500 nm, where neither ferro- nor ferricyanide absorbs. Consistent with oxygen ligation the cluster potential was found to decrease by ∼30 mV: $E^0_{native} = 345 \pm 10$ mV and $E^0_{Cys77Ser} = 315 \pm 10$ mV at 298 K in 10 mM phosphate (pH 7.5). The modest change in potential was accompanied by a more dramatic change in the thermodynamicparameters: for native, $\Delta H = -32.2$ kcal mol^{-1}, $\Delta S = -81.4$ cal K^{-1} mol^{-1}; and for Cys77Ser, $\Delta H = -19.4$ kcal mol^{-1}, $\Delta S = -40.7$ cal K^{-1} mol^{-1}. These results support an isoequilibrium relationship between reaction enthalpy and entropy,[8] with a significantly more positive reduction entropy for the mutant offsetting a less favorable enthalpy term.

The NMR spectra of the reduced and especially the oxidized mutant HiPIPs displayed prominent changes in the paramagnetic regions relative to recombinant native (Figure 1). In the reduced protein, hyperfine-shifted resonances attributed to Ser77 and Cys46 β-CH protons are observed to move relative to the corresponding cysteine signals in reduced native HiPIP (Figure 1A).[9] The assignment of the β-CH proton on Ser77 to signal w is supported by the distinct temperature dependence of that signal (supporting information). In the oxidized mutant, there is evidence for a significant perturbation of the electronic structure of the cluster.[10] Figure 1B contrasts the NMR spectra obtained for oxidized native and mutant HiPIP. The latter displays remarkable changes in the chemical shifts of resonances and their temperature dependence, which precludes specific assignment by inspection. However, one upfield signal appears to shift into the diamagnetic region (Figure 1B), consistent with a change in the equilibrium distribution of the ferric and mixed valence sites in the oxidized cluster, as recently discussed for HiPIP I from *E. halophila*.[11]

A significant perturbation of the electronic structure and stability of the oxidized cluster is consistent with the unusual behavior observed in the EPR spectrum of the oxidized mutant (Figure 2). Immediately after oxidation, the spectrum of the Cys77Serprotein (Figure 2, upper) resembles that of the native and shows the additional features that have previously been ascribed to minor HiPIP solution species, differing in either their aggregation state or electronic configuration.[10,12] However, the oxidized cluster is unstable at ambient temperature and over a period of ∼2−3 h rearranges to a form which gives rise to a distinct spectrum that bears a striking resemblance to that of a [Fe_3S_4] cluster (Figure 2, lower),[12] with a sharp feature centered around $g = 2.02$.[13] Further work will be required to fully characterize this novel species.

Conclusions. The influence of oxygen ligation on [Fe_4S_4] cluster properties has been examined. In the reduced state, there is evidence for only minor perturbations to the electronic structure of the cluster, as indicated by the similar pattern of NMR chemical shifts relative to native HiPIP. In contrast, there appears to be a stronger electronic interaction between the hard O-donor of the Ser ligand and the oxidized cluster; consequently, the oxidized Cys77Ser mutant shows rather dramatic changes in both its NMR and EPR properties. *Nevertheless, substitution of a sulfur thiolate for an oxygen ligand does not lead to a significant change in the* E^0 *for the* [Fe_4S_4]$^{3+/2+}$ *couple,* although there is a large change in the enthalpic and entropic contributions. Such an isoequilibrium relationship implies that the mutant differs from the native only with regard to the electronic

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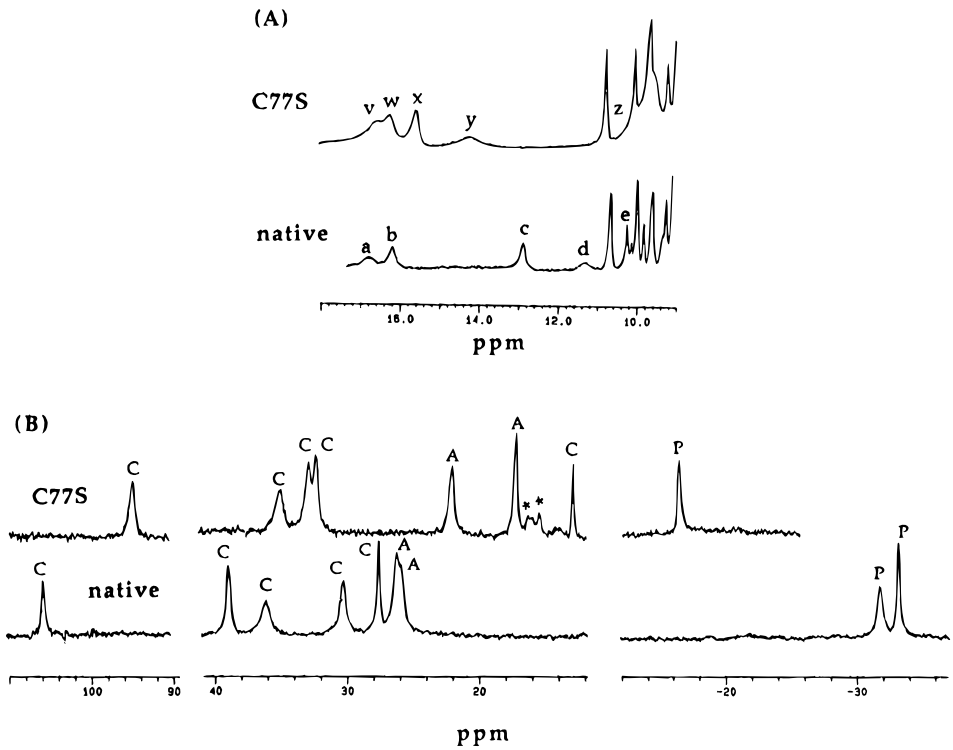

Figure 1. (A) Hyperfine-shifted resonances in the downfield region of the 500 MHz ^{1}H NMR spectra of reduced recombinant native and Cys77Ser mutant HiPIP measured at 303 K using a SUWEFT pulse sequence.[1,11] The labeled resonances all show anti-Curie behavior. For native HiPIP, resonances a−e correspond to β-CH protons on Cys43, Cys63, Cys77, Cys46, and Cys46, respectively (from ref 10). Resonances v−z are tentatively assigned to the equivalent protons on Cys43, Ser77, Cys63, Cys46, and Cys46, respectively, by comparison of line shape and temperature dependence. The temperature dependence of signal w in VT plots is significantly steeper than that of the remaining resonances (supporting information). Samples contained 1−2 mM protein in 10 mM sodium phosphate, 0.1 M NaCl at pH 6.0, and contained 10% (v/v) D_2O. (B) 500 MHz NMR spectra of oxidized native and Cys77Ser HiPIP at 303 K. Temperature-dependent behavior is denoted by C (Curie), A (anti-Curie), and P (pseudo-Curie) as previously defined.[10b] Samples were oxidized with excess potassium hexachloroiridate. Minor peaks (*) are from the reduced protein.

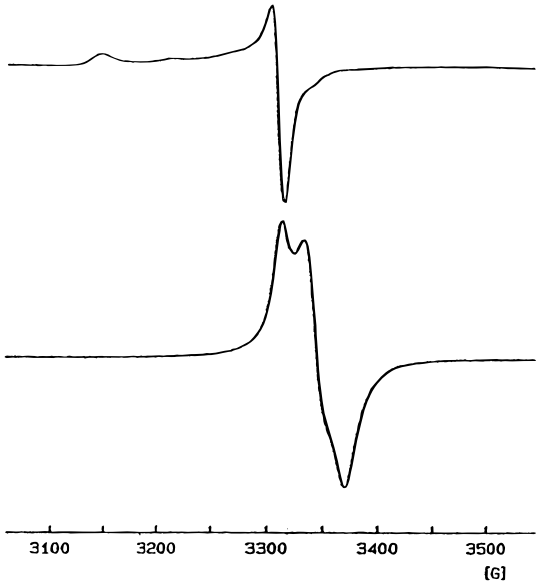

Figure 2. EPR spectra in the $g = 2$ region of the oxidized Cys77Ser mutant HiPIP obtained immediately after oxidation (upper) and after sitting for 2−3 h at ambient temperature (lower). Spectra were obtained at X-band at 20 K on argon-purged samples (4 mM protein in 100 mM phosphate, 100 mM NaCl buffer, pH 7.5) using a Bruker ESP 300 spectrometer equipped with an Oxford liquid helium cryostat. Instrumental parameters: frequency, 9.45 GHz; receiver gain, 1×10^4; modulation amplitude, 2.5 G; power, 2 mW.

properties of the cluster,[8] with no significant structural perturbation of the surrounding peptide. The minimal change in E^0 is consistent with the view that dipoles from backbone amides are a key factor in defining the cluster potential.[14] Since only the Cys77Ser mutant resulted in formation of a stable cluster, we conclude that the iron center must be ligated to achieve a stable assembly. The other side chains tested (Cys77Ala, -Asp, and -Tyr) did not yield stable proteins since the ligands were either electronically or sterically incapable of binding to the vacant coordination site on iron. There is evidence for instability of the oxidized cluster over a period of a few hours, resulting in formation of a new, but unstable cluster species. The selection of Cys rather than Ser as a cluster ligand would appear to result more from the intrinsic stability of thiolate coordination than from modulation of the reduction potential.

Acknowledgment. Supported by the National Science Foundation (Grant CHE-8921468). JAC is a Fellow of the Alfred P. Sloan Foundation, a Camille Dreyfus Teacher-Scholar, and a National Science Foundation Young Investigator. We thank Russ Hille and Craig Hemann for assistance in obtaining EPR spectra, and a reviewer for helpful comments.

Supporting Information Available: Plots of the temperature dependence of hyperfine-shifted signals for reduced and oxidized Cys77Ser mutant HiPIP (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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