A Gallium-Substituted Cubane-Type Cluster in *Pyrococcus furiosus* Ferredoxin

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Received April 25, 2001

Iron−sulfur (Fe-S) clusters are ubiquitous in nature and play a variety of roles including electron transfer and catalysis.[1] One of the best studied is the ferredoxin (Fd) from *Pyrococcus furiosus* (*Pf*). *Pf* Fd contains a single [4Fe-4S] cluster that is coordinated to the polypeptide chain by three cysteinyl and one aspartyl ligand. The high stability of this protein made it an extremely useful model system to obtain various cluster types. For example, the native 4Fe cluster is readily converted in vitro to a [3Fe-4S] form by oxidative loss of the Fe atom ligated by the aspartyl ligand.[2] Interestingly, there are no significant differences between the three-dimensional structures of *Pf* Fd with a [3Fe-4S] cluster or a [4Fe-4S] cluster.[3,4] Moreover, addition of various types of metal ion (M) to the 3Fe-form yields various mixed-metal clusters [M3Fe-4S] within this protein.[5] However, there have been very few reports on replacing all the Fe atoms in a metal-containing center. Substitution of Cd(II), Co(II), and Ru(II) into [2Fe-2S] and 2[4Fe-4S] Fds was reported but the characterization of these products did not conclusively determine the nature of the resulting metal centers.[6] Isomorphous replacement of ferric for gallium-(III) atoms in a [2Fe-2S] putidaredoxin converted it into a protein containing a [Ga-4S] center, effectively a rubredoxin-like metal center.[7] The first example of a [2Ga-2S] Fd was prepared from *Anabaena* 7120 Fd.[8] The tertiary structures of the native proteins in the latter two instances were maintained. Here we provide evidence for the first characterized all-Ga cubane-type cluster in an Fe-S protein.

The wild-type and D14C mutant forms of *Pf* Fd were used in this study. The mutant provides the classical four cysteinyl ligation to the [4Fe-4S] cluster. The two proteins were prepared as described previously.[9] The Ga-substituted forms were prepared by reconstitution of the apoproteins[10] essentially following the protocol of Vo et al.[8] The substitution of gallium for iron in metalloproteins is very useful for NMR studies because of the diamagnetic nature of gallium. The coordination chemistry and the ionic radii of gallium and iron are similar, which allows the substitution to occur without dramatic changes in the geometry of the active site of the protein.[8] Ga-substituted [2Fe-2S] Fds have been examined by NMR spectroscopy, but NMR cannot determine metal atom stoichiometry.[8,11] We show herein using Ga-substituted *Pf* Fd that accurate stoichiometries and structural information can be obtained using the complementary techniques of ESI-FTICR mass spectrometry and NMR.

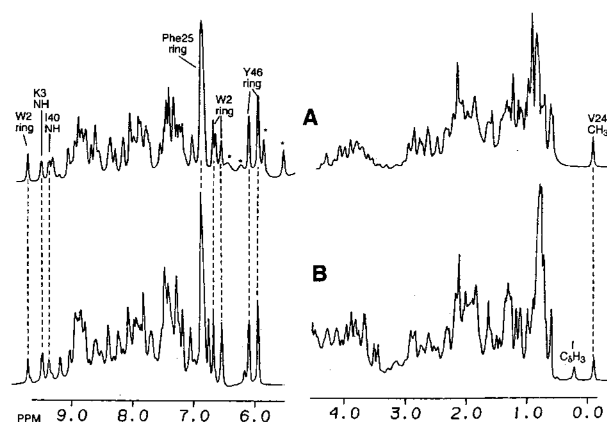

Figure 1. The 0 to 10 ppm portion of the 500 MHz ^{1}H NMR spectra of (A) wild-type *Pf* 3Fe FdN_A^{ox} and (B) the Ga-substituted Fd_A (with an intact Cys21−Cys48 bridge). The wild-type Fd exhibits additional strongly relaxed and hyperfine-shifted resonances from the three Cys in the 10−25 ppm window (data not shown). Selected resolved signals are labeled.

The 500 MHz ^{1}H NMR spectrum of the diamagnetic Ga-substituted wild-type *Pf* Fd is compared to the "diamagnetic" 0−10 ppm portion of the NMR spectrum of paramagnetic [3Fe-4S] Fd_A^{ox} (where ox indicates an oxidized cluster and the A form indicates an intact Cys21−Cys48 disulfide bridge) in Figure 1. The strong similarity of the two ^{1}H NMR spectra is striking and suggests very similar molecular structures. The NMR data are consistent with the presence of either a [4Ga-4S] or a [3Ga-4S] cluster. The apparent increase in the intensity near 8.8 and 7.5 ppm in the peptide NH spectra window and near 1 ppm in the methyl region in diamagnetic Ga-substituted Fd_A relative to paramagnetic [3Fe-4S] Fd_A^{ox} is due to the suppression by cluster paramagnetism of these resonances in the cluster ligating loop and the turn involving the last cluster ligand in the latter species. Comparison of the TOCSY spectrum of Ga-substituted Fd_A (not shown) with that of [3Fe-4S] Fd_A^{ox} on the basis of nearly identical NH shifts and TOCSY connectivity readily leads to the assignment

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(10) *Pf* Fd (60 mg; 10 mL) was denatured with HCl (1 mL; 11.6 M) at 60 °C, collected by centrifugation, and resuspended in 30 mL of Tris•Cl (0.5 M; pH 8.0). The denaturation steps were repeated three times. The apo-protein was resuspended in 30 mL of Tris•Cl (0.5 M.; pH 8.0) and DTT (1 mM). Sequentially, Na_2S then $Ga(NO_3)_3$ (10-fold molar excess of each) were added dropwise with stirring and left overnight at 4 °C. The protein was loaded onto a Pharmacia Q-HP column (2.6 × 10 cm) and washed with 2 column volumes of 50 mM Tris•Cl (pH 8.0). The Ga-substituted protein was eluted with a gradient (10 column vols) from 0 to 0.6 M NaCl in the same buffer and was concentrated by ultrafiltration (YM-3, Amicon). The sample was applied to a G-75 gel filtration column (3.5 × 60 cm), eluted with 50 mM sodium phosphate, pH 8.0, and concentrated by ultrafiltration.

10.1021/ja0160795 CCC: $20.00 © 2001 American Chemical Society
Published on Web 07/24/2001

of 40 of the 53 residues (not shown, see Supporting Information) previously assigned in the latter complex.[3] The NH shift differences are ≤0.05 ppm for residues inconsequentially relaxed in [3Fe-4S] Fd_A^{ox}, supporting in more detail a conserved structure relative to wild-type. Several broadened AMX spin systems, two Ala and a prominent Ile with methyl at 0.3 ppm (see Figure 1), are observed for which there were no analogues in [3Fe-4S] Fd_A^{ox}, and must arise from the cluster ligands and cluster loop residues. The line broadening of cluster ligand signals due to interconversion between two species in Ga-substituted Fd is similar to that observed for the same paramagnetic shifted and relaxed signals in WT [3Fe-4S] Fd[3] and has been shown to arise from interconversion of alternate orientations of the disulfide bridge.[4] The conservation in the overall folding between the two forms is particularly apparent in the highly conserved shifts for the three aromatic residues, Trp 2, Phe 25, and Tyr 46, and the presence of an intact cluster is supported by the large tertiary structural shift seen in the upfield shift for the $C_\delta H_3$ of one of the two cluster loops Ile 12 or Ile 16 in the methyl region near 0.3 ppm. Clearly, the 3Fe–Fd and the Ga-substituted Fd have virtually superimposable structures.

The metal atom stoichiometry of the Ga-substituted Fd was determined using electrospray ionization Fourier transform ion cyclotron resonance (FTICR) mass spectrometry. The validity and utility of mass spectrometry for characterizing the stoichiometry of metal clusters in metalloproteins has been established.[12] The ESI-FTICR mass spectrum[13] of the Ga-substituted D14C Fd exhibits a major peak corresponding to the [3Ga-4S] stoichiometry in the negative ion mode, while positive ionization exhibits a series of sulfur losses from a [3Ga-4S]-containing peak, analogous to those observed for [3Fe-4S] Fd.[12] By comparison to the iron–sulfur protein data, the stoichiometry of the metal center of Ga-substituted protein can be assigned as [3Ga-4S]. The ESI mass spectra for wild-type *Pf* Fd containing intact [3Fe-4S] and [4Fe-4S] clusters are shown in Figure 2, and are compared with the D14C Ga-substituted cluster in both positive and negative ionization modes. From the mass spectrum of the $[3Fe\text{-}4S]^{1+}$ protein cluster obtained in the positive mode (Figure 2d), a monoisotopic mass of 7455.92 Da was derived (assuming a disulfide bond between residues C21 and C48), which compares favorably to the calculated mass of 7455.93 Da for the neutrally charged protein + [3Fe + 4S]. From Figure 2f, a monoisotopic mass of 7511.84 Da was derived (with a disulfide bond between residues C21 and C48) for the [4Fe-4S]-containing protein compared to the calculated mass of 7511.87 Da for the neutral protein + [4Fe + 4S]. The apparent mass for the major peak in the Ga-substituted D14C Fd spectrum, Figure 2e, is 7450.96 Da and corresponds to the neutrally charged protein + [3Ga + 3S]. The monoisotopic mass for the D14C protein containing a [3Ga-3S] cluster with the remote intact disulfide bond is 7450.92 Da (with a C21–C48 disulfide bond). For all of the proteins, the positive mode ESI mass spectra are characterized by one or two charge states, while the negative ion mass spectra exhibit 5–6 charge states.

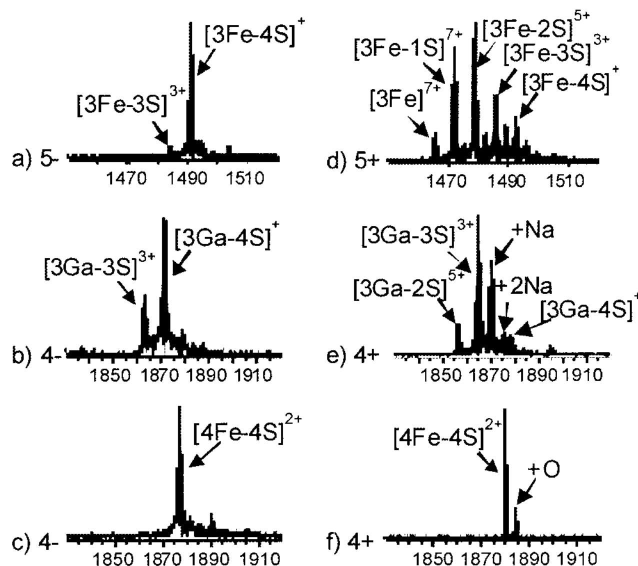

Figure 2. ESI-FTICR mass spectra of *Pf* Fd acquired in both positive and negative ionization mode: (a) [3Fe-4S]-containing Fd, negative ion mode, (b) Ga-substituted Fd, negative ion mode, (c) [4Fe-4S]-containing Fd, negative ion mode, (d) [3Fe-4S]-containing Fd, positive ion mode, (e) Ga-substituted Fd, positive ion mode, and (f) [4Fe-4S]-containing Fd, positive ion mode. The shift to lower mass-to-charge in the negative ion mass spectrum compared to positive ion mode is typically observed for nondenaturing conditions. The charge states for both positive and negative ions in the mass spectra are listed to the left of the mass axis.

Hence, the mass spectrum of the Ga-substituted D14C protein resembles that of the [3Fe-4S] Fd rather than its [4Fe-4S] counterpart both in positive and negative ionization modes. These data complement the NMR results which show a strong similarity between the spectra of the [3Fe-4S] and Ga-substituted forms of the wild-type protein. Taken together, the mass spectral data and the NMR data are consistent with a stoichiometry for the metal center of [3Ga-4S] rather than 4Ga-4S] while maintaining almost identical tertiary structures. While reconstitution of the apo-form of *Pf* Fd (both wild-type and D14C) with Fe(II) yields exclusively a protein containing a [4Fe-4S] cluster, we show here that when Ga(III) replaces Fe(II), the protein instead contains a [3Ga-4S] center. The reasons for this are unclear; nevertheless, the present case represents the first example of a Ga-substituted *cubane-type* cluster protein with sufficient stability to permit detailed structural characterization.

Acknowledgment. We gratefully acknowledge funding provided by the National Science Foundation: CHE 9984579 (I.J.A.), MCB 9600759 (GNL), and MCB 9904624 (MWWA). We thank Michael Johnson for heplful discussions.

Supporting Information Available: Comparison of chemical shift for selectedresidues of *Pf* 3Fe F_A^{ox} with those of *Pf* 3Ga Fd_A (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0160795

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