Preparation and Characterization of [2Ga-2S] *Anabaena* 7120 Ferredoxin, the First Gallium−Sulfur Cluster-Containing Protein

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Abstract: The preparation and characterization of the [2Ga-2S] analog of the [2Fe-2S] ferredoxin from vegetative cells of the cyanobacterium *Anabaena* 7120, a prototypical plant-type ferredoxin, is described. The novel metal-substituted analog was obtained through constitution of the apoprotein with Ga(III) and sulfide anion in argon-saturated aqueous buffer. The replacement product migrated identically with the diferric protein on a 15% native PAGE gel and contained 2 atom equiv of gallium and sulfide per mole of protein, according to inductively coupled plasma and colorimetric analysis, respectively. The EXAFS spectrum of the gallium−sulfide constituted protein indicated the presence of a [2Ga-2S] cluster with structural features similar to those of [2Fe-2S] clusters. Cross peaks from nuclei that were not affected by the paramagnetism of the iron−sulfur cluster in two-dimensional ^{1}H NMR spectra of the [2Fe-2S] ferredoxin appeared at chemical shifts essentially equivalent to those arising from analogous nuclei in the spectra of the [2Ga-2S] protein. Spectra of the gallium derivative, however, also possessed additional resonances; for example, 19 additional $H^{\alpha}-H^{N}$ cross peaks were observed in the fingerprint region of the DQF-COSY spectrum of the [2Ga-2S] protein taken in H_2O at 298 K. Sequential assignment of the resonances confirmed that the additional cross peaks originated from amino acids in the vicinity of the metal−sulfur cluster, which were hyperfine-shifted or broadened beyond detectability in the iron−sulfur protein spectra. Preliminary analysis of the NMR spectra indicated that the structural features of the gallium−sulfide constituted protein possessed many similarities to those of the [2Fe-2S] protein in the crystalline state. Gallium-substituted analogs of iron−sulfur proteins should find utility in characterization of the structural and functional features of the ferric forms of the native iron−sulfur proteins using conventional NMR techniques.

Metal ion substitution is a valuable technique for investigating structure−function relationships in metalloproteins. Replacement of the native metal of a protein with a metal ion with useful spectroscopic features, for example, can provide insight into the coordination environment of the metal,[1] while other metals can facilitate structural investigations.[2] Iron−sulfur proteins are a class of metalloproteins that contain iron and inorganic sulfur in the form of clusters of different nuclearity, which are bound to the polypeptide by side chain atoms of amino acids. Although iron−sulfur proteins constitute the largest and most varied group of metalloproteins,[3] examples of isomorphous replacement of the iron ions of these molecules with other metals are rare. While accounts of the exchange of the iron ions of [2Fe-2S] and [4Fe-4S] ferredoxins with Cd(II),[4] Co(II),[5] and Ru(II)[5] have appeared, the homogeneity of the products and the exact compositions of the product clusters were not conclusively established.

Ga(III) forms complexes that are frequently isomorphous with the analogous Fe(III) compounds as a result of the two ions' similarities in ionic radii and coordination chemistry.[6] Due to its diamagnetic nature, Ga(III) is an attractive substitute for the paramagnetic Fe(III) ions of iron-containing coordination compounds for ^{1}H NMR applications.[7] The nuclear spin of gallium ($I = {}^3/_2$ for ^{71}Ga and ^{69}Ga)[8] has enabled heteronuclear NMR studies to be performed on a Ga(III)-substituted mononuclear iron protein.[9] In addition, redox-inactive Ga(III)-substituted proteins would theoretically form inert complexes with the redox partners of the native iron proteins, allowing investigation of the structural and dynamic features of such species. Self-assembly of [2Ga-2S] clusters has been demonstrated when Ga(III) salts are combined with thiol ligands in the presence of sulfide.[10] Although a derivative of a bacterial [2Fe-2S] ferredoxin with a single gallium ion bound to the four cysteine thiol ligands has been prepared,[11] the successful formation of proteins containing gallium-sulfur *clusters* has not been previously accomplished. We now report the preparation and characterization of the [2Ga-2S] analog of the [2Fe-2S] ferredoxin from vegetative cells of the cyanobacterium *Anabaena* 7120,[12] the first well-characterized metal-substituted

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iron−sulfur protein with cluster nuclearity identical to that of the native protein.[13]

Materials and Methods

General. Unless otherwise noted, all starting materials and solvents were obtained from commercial suppliers and used without further purification.

Preparation. Native [2Fe-2S] *Anabaena* ferredoxin was obtained through expression of the gene in *Escherichia coli* strain BL21(DE3) pLysS (Novagen) as described by Vidakovic et al.[14] The apoferredoxin was prepared according to the method of Meyer as follows.[15] A solution of the ferredoxin (5.5 mg/mL in 100 mM Tris HCl, pH 8.0) was degassed for 30 min by blowing humidified Ar gas over the surface of the solution. Concentrated HCl was added to the solution to a final concentration of 0.5 M. The solution immediately adopted a cloudy white appearance, and the reaction was allowed to proceed at room temperature with continuous stirring under an Ar flow for 1 h. The solution was centrifuged (10 min at 16 000 rpm), and the white pellet was quickly rinsed with distilled water. The resulting precipitate of the apoferredoxin was dissolved in degassed 0.5 M Tris HCl, pH 8.0, containing 1 mM dithiothreitol (DTT) to a final concentration of 5 mg/mL, assuming complete recovery of protein. The precipitation procedure was repeated to ensure complete removal of Fe(III) and sulfide, and the pellet was resuspended in degassed 0.5 M Tris HCl, pH 8.0, 1 mM DTT to a final protein concentration of 2 mg/mL.

To the solution of the apoferredoxin was added a freshly prepared solution of 0.1 M Na_2S (10 equiv) and 0.1 M $GaCl_3$ (10 equiv). The resulting clear solution was stirred under Ar at 4 °C for 15 h. The constituted protein was then separated from excess reagent by passage through a Q-Sepharose FF column, eluting with 20 mM Tris HCl, pH 7.6, 0.5 M NaCl. Fractions which contained protein material that migrated identically to the diferric protein on a 15% PAGE gel were pooled and concentrated in an Amicon apparatus. The buffer was exchanged with 50 mM Tris HCl, pH 8.0 for storage, or with 50 mM sodium phosphate, pH 7.1 for NMR experiments.

Absorbance Spectroscopy. The absorption spectra of the proteins and other samples were obtained at room temperature using an HP 8452A diode array spectrophotometer. CD spectra of ferredoxins in 50 mM sodium phosphate buffer pH 7.1 at room temperature were recorded on Jasco-J700 spectropolarimeter. Raw CD spectra were subjected to line smoothing with the software supplied by the manufacturer.

Microanalysis. Concentrations of diferric *Anabaena* ferredoxin solutions were determined by absorption spectroscopy using an extinction coefficient at 422 nm of 9200 M^{-1} cm^{-1}.[16] Concentrations of gallium-substituted ferredoxin solutions were determined using an extinction coefficient at 276 nm of 7100 M^{-1} cm^{-1}, calculated from the number of tyrosine residues in the sequence of the apoprotein[17] (five; *Anabaena* ferredoxin contains no tryptophan residues) and the extinction coefficient of free tyrosine (1420 M^{-1} cm^{-1}).[18] Protein concentrations were also determined using the Coomassie Plus protein assay reagent manufactured by Pierce (Rockford, IL). Reaction of proteins with dithiobis(nitrobenzoic acid) (DTNB) was carried out as described.[19] Acid-labile sulfide content of the protein solutions was determined by the methylene blue method reported by Beinert.[20] Protein to sulfide ratios were calculated relative to a standard solution of Na_2S prepared freshly prior to protein analysis. Gallium content was determined by inductively coupled plasma analysis by Galbraith Laboratories.

Mass Spectrometry. Electrospray mass spectra were obtained by the Washington University Mass Spectrometry Resource, St. Louis, MO. Mass peaks were detected in the positive mode using a magnetic sector instrument equipped with a VG organic nebulizer assisted electrospray source using nitrogen as the nebulizer gas and bath gas. An 8 kV spray voltage was used. Samples were suspended in 1% formic acid/50% methanol at a concentration of approximately 50 μM and were injected at a rate of 10 μL/min through a 20 μL injection loop. Ten scans in the 100−2600 Da (dalton) range were scanned and averaged to obtain final spectra.

EXAFS Spectroscopy. EXAFS spectra were recorded on 5 mM protein samples as frozen glasses at 77 K at the Brookhaven National Laboratory Synchrotron Facility by Dr. James Penner-Hahn, Department of Chemistry, University of Michigan, Ann Arbor, MI. Experimental details regarding data accumulation and analysis will be published separately.

NMR Spectroscopy. All samples contained 3−5 mM protein in 0.5 mL of 50 mM phosphate buffer, pH 7.1 that contained 10% D_2O. Samples in D_2O in the same buffer were prepared by lyophilization of the H_2O sample and resuspension in 0.5 mL of D_2O (99.8 atom % D).

^1H NMR spectra were recorded on a Bruker AMX-600 spectrometer at 288, 298, or 308 K. Data were processed using FELIX 95 software (MSI). Water suppression was achieved through presaturation of the H_2O signal during the relaxation delay. TPPI was used for performing phase-sensitive DQF-COSY,[21] NOESY,[22] and TOCSY[23] experiments. Mixing times of 60 and 80 ms were used for TOCSY[23] spectra, and 80 and 160 ms for NOESY spectra. All spectra were referenced to the chemical shift of the residual H_2O signal. In general a total of 64 transients were recorded with a relaxation delay of 2−3 s and a spectral width of 8.1 kHz. Five hundred twelve increments of 2 K data points were collected in each two-dimensional experiment and were zero-filled so that spectra with 2K × 2K data points were obtained. A window function of a 60° shifted sine bell was used for the DQF-COSY spectra, and a 90° shifted squared sine bell was used for the NOESY and TOCSY spectra.

Results

Native *Anabaena* ferredoxin was obtained through expression of the gene in *E. coli*.[14] The apoprotein was prepared according to the method of Meyer.[15] It was found that the success of the constitution was critically dependent on the method of apoprotein preparation. When the apoprotein was prepared using trichloroacetic acid instead of HCl, the constitution reaction resulted in the formation of several products, according to gel electrophoresis.

The Ga(III)-substituted protein was obtained through constitution of the apoprotein with Ga(III) and sulfide in aqueous solution. The progress of the constitution reaction was monitored by native PAGE. This sensitive technique can detect minor differences in the overall charge and conformation state of a protein and thus is useful for assessing the structural similarities of related proteins. Ferredoxins are highly acidic proteins,[24] and *Anabaena* ferredoxin migrates close to the bromophenol marker on a 15% native PAGE gel. Apoferredoxin is unstructured according to CD spectroscopy,[25] and it migrates much more slowly on the gel than the [2Fe-2S] form (Figure 1).

Constitution of the apoferredoxin was carried out with $GaCl_3$ and Na_2S in argon-saturated Tris HCl buffer in the presence of DTT at 4 °C. Use of $Ga(NO_3)_3$ in place of $GaCl_3$ gave identical

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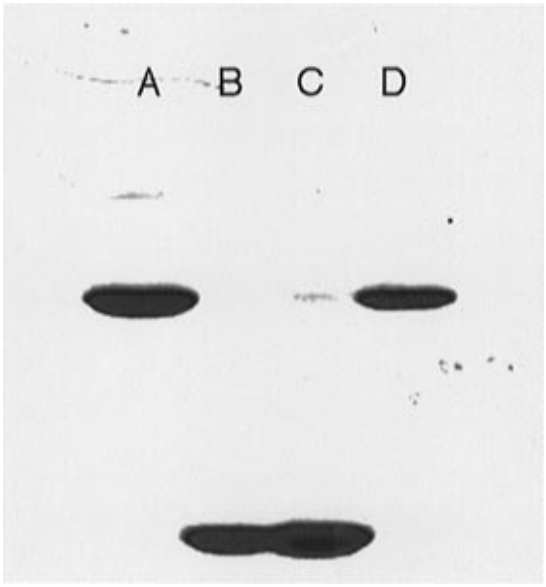

Figure 1. Native PAGE (15%) of *Anabaena* ferredoxins. Lane A: apoferredoxin. Lane B: native oxidized [2Fe-2S] ferredoxin. Lane C: constitution product of apoferredoxin with $GaCl_3$ and Na_2S. Lane D: apoferredoxin constituted with $GaCl_3$ as in lane C, but without Na_2S.

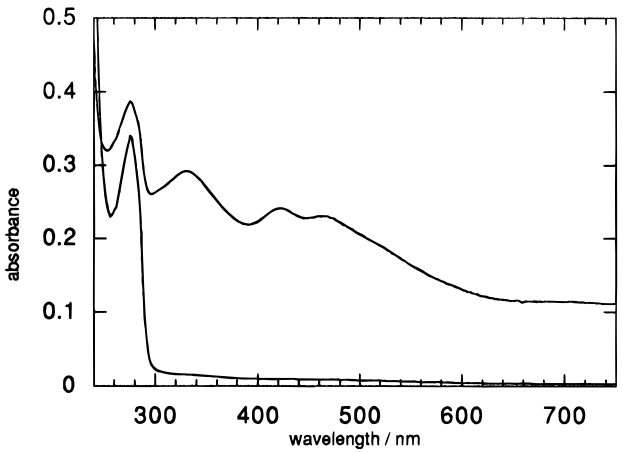

Figure 2. Absorbance spectra of native oxidized [2Fe-2S] *Anabaena* ferredoxin (top) and Ga(III)−sulfide constitution product (bottom) at room temperature in 20 mM Tris HCl, pH 8.0.

results. The reaction mixture contained a product that behaved identically to the native diferric ferredoxin on the 15% native PAGE gel and that was distinct from the apoprotein (Figure 1). When the apoferredoxin was exposed to the constitution conditions without added Na_2S, the observed band migrated identically to that for the apoprotein and was distinct from that for the gallium and sulfide constitution product.

The crude constitution reaction mixture was applied to an anion exchange column, and proteins were eluted with Tris buffer containing 0.5 M NaCl. Fractions containing protein product were individually analyzed by native PAGE (15%), and those that contained material that migrated identically to the native ferredoxin were pooled and concentrated. The absorbance spectrum of the Ga(III)-substituted product was transparent in the visible region, indicating the absence of iron−sulfur centers (Figure 2). The concentration of the polypeptide moiety was estimated by a dye-binding assay, as well as by absorbance spectroscopy. An extinction coefficient at 278 nm of 7100 M^{-1} cm^{-1} for the protein was calculated on the basis of the five tyrosine residues in the sequence.[17] Additionally the protein concentration was determined by NMR spectroscopy by integration of well-resolved ^1H signals against an internal standard.

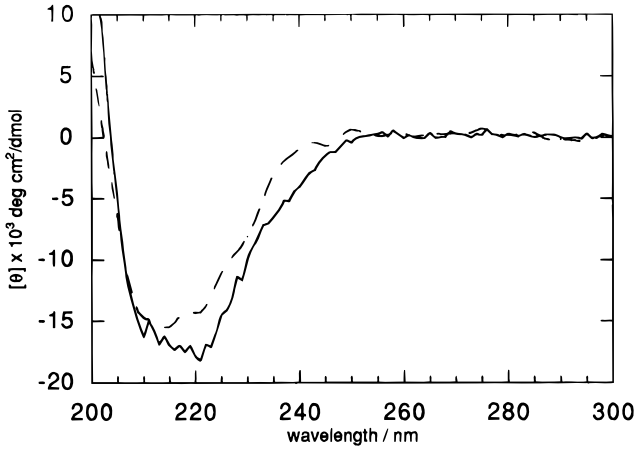

Figure 3. UV CD spectra of oxidized [2Fe-2S] *Anabaena* ferredoxin (solid line) and Ga(III)−sulfide constitution product (dashed line) at room temperature in 20 mM Tris HCl, pH 8.0.

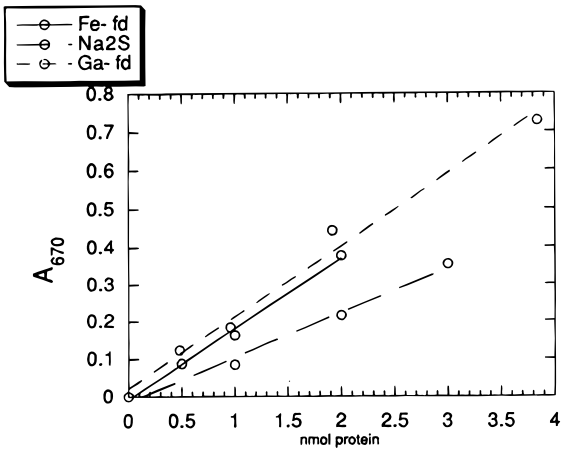

Figure 4. Colorimetric determination of sulfide in *Anabaena* ferredoxins, as measured by absorbance of methylene blue at 670 nm, against a standard of Na_2S.

All methods gave similar values for the protein concentration, allowing a yield of 60% for the constitution procedure to be calculated.

The ultraviolet circular dichroism spectrum of the product was essentially identical to that of native *Anabaena* ferredoxin (Figure 3). Both spectra displayed strong bands with negative Cotton effects at about 215 nm, indicative of significant β structure. Furthermore the CD spectrum of the Ga(III)-substituted protein was distinct from that of the apoprotein, which is suggestive of a random conformation.[25]

The inorganic element content of the Ga(III)-substituted *Anabaena* protein was determined by microanalysis. Colorimetric analysis[20] revealed that the gallium substitution product incorporated an equivalent amount of acid-labile sulfide per mole of protein as did the native ferredoxin (Figure 4). Measured against a standard Na_2S solution, the Ga(III) and sulfide constituted ferredoxin was found to incorporate 1.7 ± 0.2 equiv of acid-labile sulfur per protein equivalent. Inductively coupled plasma analysis indicated that the product contained 1.8 ± 0.2 equiv of gallium per mole of protein.

The Ga(III)-substituted protein was inert to the thiol-specific reagent DTNB,[19] indicating the absence of free cysteine thiol groups in the protein. The four cysteine residues in the sequence of *Anabaena* ferredoxin are coordinated to the [2Fe-2S] cluster in the native protein.[26] Since the constitution reaction was carried out under reducing conditions, the lack of free thiol

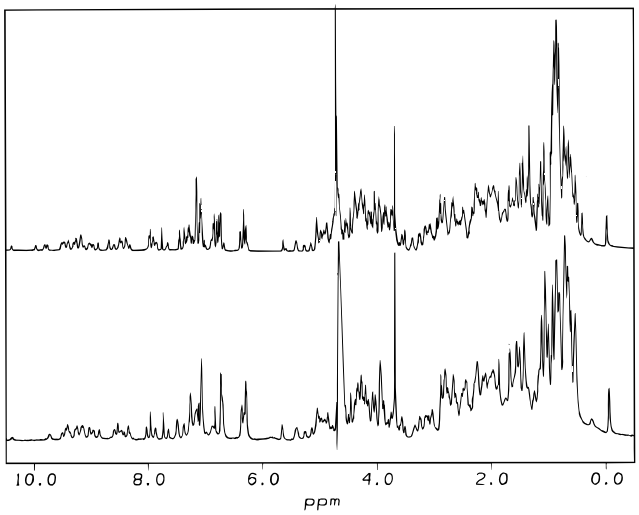

Figure 5. One-dimensional ^{1}H NMR spectra of 3 mM samples of oxidized [2Fe-2S] *Anabaena* ferredoxin (bottom) and Ga(III)−sulfide constitution product (top) taken at 298 K in 50 mM phosphate, pH 7.1 in D_2O.

groups implied that all four cysteine residues of the Ga(III)-substituted protein were bound to metal ions.

The electrospray ionization mass spectrum (EIMS) of the gallium ferredoxin (1% formic acid) displayed a peak with a mass of 10 888.3, which closely corresponded to the mass expected for the [2Ga-2S] protein (10 889). In addition, a peak originating from the apoprotein was observed under these conditions (10 693.4; calculated mass 10 687).

Convincing evidence for the presence of a [2Ga-2S] cluster in the gallium-substituted ferredoxin was obtained from X-ray absorption fine structure (EXAFS) spectroscopy. The Fourier transform of the Ga-EXAFS spectrum of the Ga(III)-substituted *Anabaena* ferredoxin had an appearance similar to those of [2Fe-2S] ferredoxin Fe-EXAFS spectra.[27] Two peaks assignable to S and Ga shells at approximately the same distances seen for S and Fe shells in EXAFS spectra of [2Fe-2S] clusters were clearly distinguishable. The Ga−S peak height was typical of MS_4 ligation, and the Ga−Ga peak was only slightly smaller than the Fe−Fe peak in authentic [2Fe-2S] sites.[28]

The conformational features of the polypeptide of the Ga(III)-substituted *Anabaena* ferredoxin were assessed by NMR spectroscopy. In NMR spectra of oxidized plant-type [2Fe-2S] ferredoxins, signals from atoms closer than 9 Å to the iron−sulfur cluster are shifted outside the diamagnetic region and/or broadened beyond detectability as a result of interaction with the unpaired electrons of the iron−sulfur cluster.[29] The diamagnetic regions of the one-dimensional ^{1}H NMR spectra of the native and gallium-substituted ferredoxins are shown in Figure 5. Both spectra are closely related, with signals in the iron protein spectrum appearing at similar chemical shifts in the gallium protein spectrum.

Several differences in the appearance of the spectra of the native and gallium-substituted ferredoxins, however, were apparent. First, the gallium protein spectrum displayed narrower line widths than the iron protein spectrum. Second, the diamagnetic region of the gallium protein spectrum contained more resonances than did the iron protein spectrum. These additional signals presumably originated from amino acids located in the vicinity of the paramagnetic iron−sulfur cluster of the native ferredoxin. Not surprisingly, the hyperfine-shifted resonances of the oxidized iron protein spectrum (δ 15−50 ppm)[31] were not observed in the gallium analog's spectrum.

Homonuclear two-dimensional NMR spectra of the Ga(III)-substituted and native ferredoxins also displayed exceptional similarities in appearance. For example, all $H^{\alpha}-H^{N}$ cross peaks in the fingerprint region of the double-quantum filtered COSY (DQF-COSY) spectrum of the [2Fe-2S] ferredoxin had a counterpart at an essentially identical position in the spectrum of the Ga(III)-substituted protein (Figure 6). The differences in chemical shifts of the corresponding signals were less than 0.05 ppm. The fingerprint region of the Ga(III)-substituted protein spectrum taken in H_2O at 298 K exhibited 19 additional $H^{\alpha}-H^{N}$ cross peaks that were not present in the [2Fe-2S] ferredoxin spectrum (Figure 6). The additional resonances were found to originate from amino acid residues in the vicinity of the metal cluster and represented the majority of the 20 amino acids whose signals were unaccounted for in the [2Fe-2S] protein spectrum.[29]

Sequence-specific assignment of the resonances of the Ga(III)-substituted protein spectrum was carried out by standard methods.[30] The assignment of most of the resonances was greatly aided by comparison with the spectrum of the oxidized [2Fe-2S] protein, whose diamagnetic region resonances had been previously assigned.[29] For example, the sequential H^{α}_i/H^{N}_{i+1} connectivities seen by Markley for the diamagnetic resonances in the nuclear Overhauser spectroscopy (NOESY) spectrum of the oxidized [2Fe-2S] ferredoxin were also observed for the resonances with equivalent chemical shifts in the gallium protein NOESY spectrum. This result established that those peaks that displayed identical chemical shifts in both iron and gallium ferredoxin spectra originated from the same hydrogens.

The sequence-specific assignment of those resonances that were not visible in the spectra of the diferric ferredoxin was carried out as follows. The spin systems of the additional resonances were identified using total correlation spectroscopy (TOCSY) and DQF-COSY. Sequence-specific assignments for the new resonances were then established by locating sequential connections from neighboring residues whose resonances were assigned in the NOESY spectrum of the [2Fe-2S] ferredoxin. For instance, sequential assignment of the resonances from the C-terminus of the [2Fe-2S] protein ended at Ala 82 because no cross peaks between Ala 82 and Val 81 signals were visible.[29] The resonances of Val 81 were easily identified in the gallium protein spectrum, however, on the basis of strong H^{α}_i/H^{N}_{i+1}, H^{N}_i/H^{N}_{i+1}, and H^{β}_i/H^{N}_{i+1} cross peaks to the Ala 82 NH resonance. Strong H^{α}_i/H^{N}_{i+1} and H^{N}_i/H^{N}_{i+1} cross peaks were then found between the resonances of Val 81 and Cys 80, as well as Cys 80 and Thr 79. In addition the TOCSY and DQF-COSY spectra revealed that the signals assigned to the H_{β} and H_{γ} hydrogens of Thr 79 were coupled to another hydrogen that resonated at 5.52 ppm. This resonance was assigned to the Thr 79 $O_{\gamma}H$, which possibly exchanged sluggishly with solvent due to its location in the protein interior.

Assignment of the resonances of the metal-binding loop amino acids (Pro 38 to Ala 50) was initiated from the Gly 51 signals, which appeared at equivalent positions in both iron and

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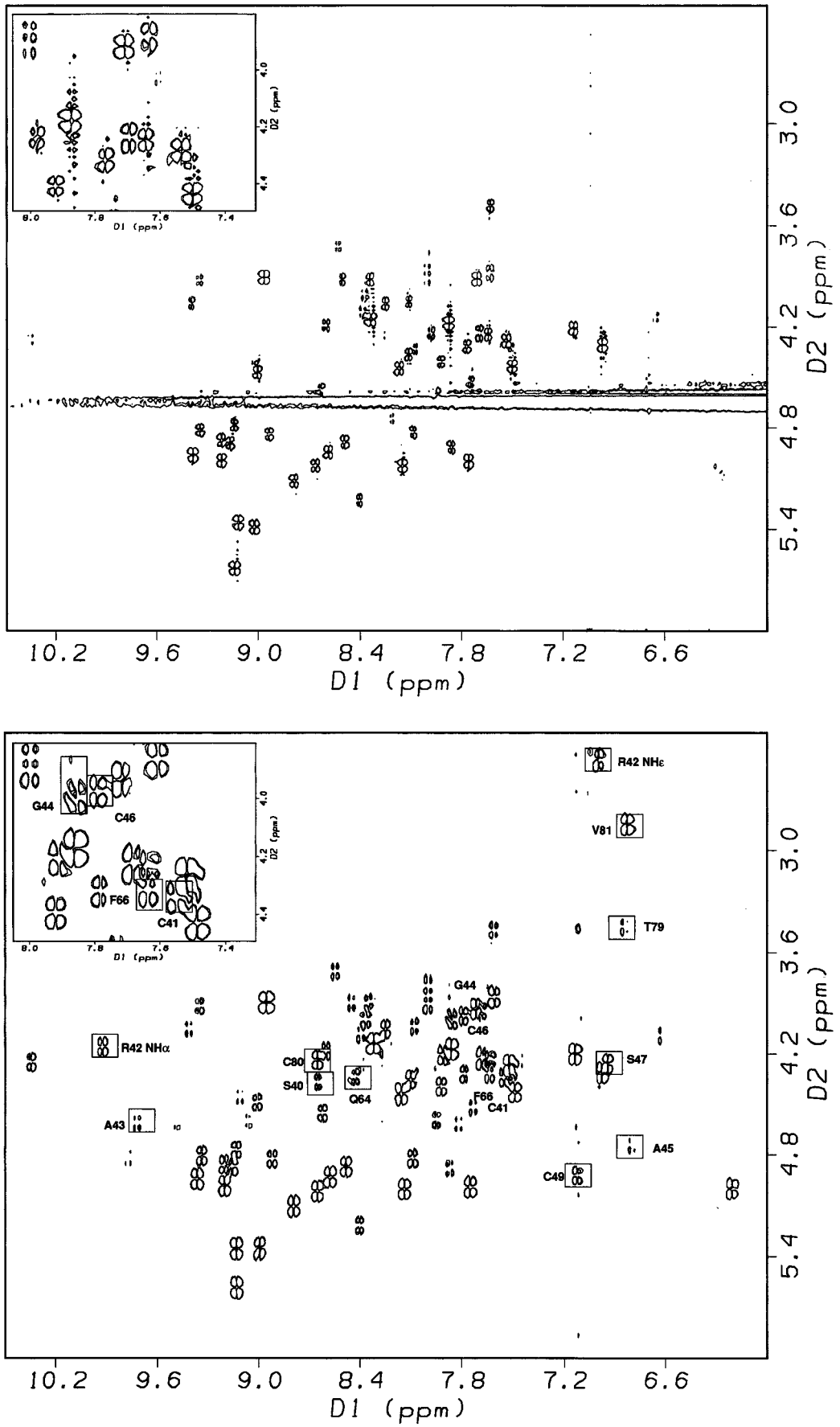

Figure 6. (A) Fingerprint region of the DQF-COSY spectrum of [2Fe-2S] *Anabaena* ferredoxin taken at 298 K in 50 mM phosphate, pH 7.1 in H_2O. (B) Fingerprint region of the Ga(III)−sulfide constituted *Anabaena* ferredoxin taken under the same conditions as in panel 6A. Expansions of sections from the centers of the spectra are shown as insets in the upper right hand corners of both spectra. Cross peaks that were not detectable in the [2Fe-2S] protein spectrum are boxed and labeled according to sequential assignment in panel B.

gallium protein spectra. Analysis of spectra obtained at two other temperatures (288 and 308 K) was necessary to locate those signals that were obscured by the H_2O resonance at 298 K. The Gly 51 NH showed NOEs to both the β-CH_3 and NH resonances of Ala 50 (the Ala 50 and one of the Gly 51 H_α hydrogens had equivalent chemical shifts). H^{α}_i/H^{N}_{i+1} and a number of H^{N}_i/H^{N}_{i+1} connectivities were then located for the stretch from Phe 39 to Ala 50.

The resonances of Ser 65 and Phe 66 were assigned starting from those of Leu 67 and Gln 64. Although both of the latter two residues displayed ^{1}H chemical shifts that were similar to those of the corresponding residues in the [2Fe-2S] protein spectrum, the signals from these residues were significantly more intense in the gallium protein spectra. Clear H^{α}_i/H^{N}_{i+1} and H^{N}_i/H^{N}_{i+1} and a number of H^{β}_i/H^{N}_{i+1} connectivities were visible among all these residues. A globally ^{15}N-labeled sample of *Anabaena* ferredoxin is currently being prepared for 2-D and 3-D heteronuclear NMR spectroscopy in order to support the sequence-specific assignments derived from homonuclear spectra and to locate residues that were not assigned from the homonuclear studies.

NMR spectra of the gallium *Anabaena* ferredoxin taken after exchange of the buffer with D_2O showed that a number of backbone amide hydrogens had not appreciably exchanged with the deuterated solvent after 100 h. The backbone amide protons that underwent slow exchange in the [2Fe-2S] ferredoxin were equally reluctant to exchange in the gallium derivative.

Discussion

Constitution of the apo form of the [2Fe-2S] ferredoxin from *Anabaena* 7120 with Ga(III) and sulfide afforded a stable product that incorporated both elements. The analytical data established a ratio of 2 equiv of gallium and acid-labile sulfide per protein molecule. The EXAFS data provided evidence for the presence of a [2Ga-2S] cluster bound to four sulfur ligands, with structural features similar to those of [2Fe-2S] clusters of ferredoxins. PAGE analysis revealed the gallium-substituted protein to have electrostatic and structural characteristics similar to those of the native [2Fe-2S] protein.

The ^{1}H NMR spectra of the gallium-substituted ferredoxin displayed high similarity to those of the oxidized [2Fe-2S] protein, with additional resonances from amino acids that were located in the vicinity of the metal cluster. For example, all sequential H^{α}_i/H^{N}_{i+1} cross peaks of the [2Fe-2S] ferredoxin NOESY spectrum displayed positions and intensities essentially identical to those of the analogous cross peaks of the [2Ga-2S] ferredoxin spectrum. In addition, the cross peaks seen in the NH−NH region of the NOESY spectrum of the [2Fe-2S] ferredoxin were present in the [2Ga-2S] protein spectrum. Hence both forms of this ferredoxin possessed similar three-dimensional structural features in solution for those regions located greater than 9 Å from the metal cluster.

Preliminary analysis of the NMR spectra of the gallium protein suggested that the conformation of the polypeptide close to the metal cluster was similar to that seen for the [2Fe-2S] protein in the X-ray structure.[26] For example, the crystal structure showed that residues Leu 78 to Val 81 form a type I β turn.[26] The NOESY spectrum of the Ga(III)-substituted protein displayed strong cross peaks between the resonances assigned to the Val 81 and Cys 80 NH's, between the Cys 80 NH and the Thr 79 NH, and between the Thr 79 H^{α} and Val 81 NH, as expected for a type I β turn.[30] A number of strong NH−NH NOEs were also seen between select residues of the metal-binding loop (for example, between Arg 42 and Ala 43; Ala 43 and Gly 44; and Ala 45, Gly 44, and Cys 46); the crystal structure of the [2Fe-2S] form reveals these pairs of hydrogens to be in close proximity. Also the Val 81 H^{α} proton of the gallium protein was strongly shielded (2.86 ppm); this observation can be explained by the proximity of this hydrogen to the Tyr 25 aryl ring, as seen in the X-ray structure of the iron analog.

The similarity of the solvent exchange parameters for the two ferredoxin forms demonstrated that the two proteins had equivalent patterns of hydrogen bonding and comparable dynamic properties. In addition, 10 of the additional $H^{\alpha}-H^{N}$ cross peaks in the Ga(III)-substituted protein two-dimensional spectra were found to persist after 100 h of exposure to deuterated buffer. The possibility that these slowly exchanging NHs are involved in NH−S hydrogen bonds[32] is currently being investigated.

Interestingly, Pochapsky and co-workers reported that when the apo form of the non-plant-type [2Fe-2S] protein putidaredoxin was constituted with Ga(III) and sulfide, a novel derivative that incorporated a single gallium atom coordinated to the four original cysteine ligands was isolated.[11] The differences between the behavior of apoputidaredoxin and the apoferredoxin prepared in this study may have originated from kinetic or thermodynamic factors. At pH > 7.4, Ga(III) is split approximately 50:1 between the soluble $Ga(OH)_4^-$ and the relatively insoluble $Ga(OH)_3$.[33] In the presence of ligands capable of metal coordination, such as Tris and DTT, Ga(III) would most likely be complexed with these species. Regardless of the most prevalent form of Ga(III) under the reaction conditions, successful constitution of the apoprotein with the [2Ga-2S] cluster would depend on the relative values of the formation constants of all of the gallium-containing species. As the logarithm of the formation constant of $Ga(OH)_4^-$ is 19.01,[34] the corresponding formation constant for the [2Ga-2S]-constituted protein could not be significantly below this value to allow isolation of Ga(III)-substituted product. Structural differences between [2Ga-2S] and [2Fe-2S] clusters, although slight,[10] may be appreciable enough to thwart formation of the [2Ga-2S] putidaredoxin. Interestingly, while a rubredoxin-like $(Cys)_4Fe^{III}$ form of the non-plant-type [2Fe-2S] ferredoxin adrenodoxin has been generated,[35] the analogous analog of any plant-type ferredoxin has not been reported. The gallium constitution procedure described here, however, is general; the [2Ga-2S] derivatives of the [2Fe-2S] ferredoxin from *Trichomonas vaginalis*[36] and the Ala45Ser mutant of *Anabaena* ferredoxin[37] have been obtained in this laboratory.

Conclusion

The [2Ga-2S] analog of the [2Fe-2S] ferredoxin from *Anabaena* has been prepared and is amenable to structural characterization by conventional NMR techniques. Preliminary analysis of the spectral features of the gallium derivative indicate that the [2Ga-2S] protein is an isostructural analog of the [2Fe-2S] protein. The detailed comparison of the three-dimensional structure of the [2Ga-2S] protein obtained by distance geometry and restrained molecular dynamics calculations using NMR constraints to that of the [2Fe-2S] ferredoxin obtained by X-ray crystallography is being actively pursued.

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Supporting Information Available: NOESY spectra of the Ga(III)-substituted protein (4 pages). See any current masthead page for ordering and Internet access instructions

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