Identification of a Key Intermediate of Relevance to Iron−Sulfur Cluster Biosynthesis. Mechanism of Cluster Assembly and Implications for Protein Folding

Kshama Natarajan and J. A. Cowan*

Evans Laboratory of Chemistry, The Ohio State University 100 West 18th Avenue, Columbus, Ohio 43210

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Proteins which carry a complex metalloprosthetic center pose interesting questions in peptide folding, since this may be accompanied by insertion or assembly of the metal cofactor. We have followed the early stages of iron−sulfur cluster assembly and backbone folding for native *Chromatium vinosum* HiPIP using $^{1}H-^{15}N$ heteronuclear single quantum coherence (HSQC) and matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry experiments. These studies suggest that the $[Fe_4S_4]$ cluster assembles through a key structural intermediate that already possesses to a large extent the secondary and tertiary structural elements associated with the native protein. This intermediate is most likely relevant to the assembly of other complex Fe/S clusters in metalloproteins and enzymes, and these studies offer insight into molecular mechanisms for biosynthetic cluster assembly.[1,2]

Heteronuclear NMR spectroscopy affords a powerful and direct tool to monitor protein folding by following the time evolution of $^{1}H-^{15}N$ HSQC crosspeaks during iron−sulfur cluster assembly, since these provide a reliable fingerprint of local and global structure. This strategy has previously been used by us in studies of cluster stability in mutant HiPIPs.[3,4] Our initial studies of $[Fe_4S_4]$ cluster assembly from apo-HiPIP in the presence of Fe^{2+}, S^{2-}, and the reducing agent DTT proved successful,[5,6] and the resulting $^{1}H-^{15}N$ HSQC spectrum resembled the native spectrum (Figure 1F). Surprisingly, spectra recorded over a time-frame of 30 min to several hours after the addition of only Fe^{2+} and DTT were found to be almost identical to the native HiPIP spectrum and were unlike the apo-form (Figure 1). Addition of Fe^{2+} led to the appearance of assignable crosspeaks in the $^{1}H-^{15}N$ HSQC spectrum, most of which were superimposable on the signals from the native spectrum (Figure 1F). Most other crosspeaks show only minor shifts ($\leq \delta \pm 0.1$ ppm) relative to the corresponding signals in the native spectrum. The similarity in the position and pattern of >85% of the crosspeaks, which extends over the entire sequence of residues, provides a powerful affirmation of the structural resemblance of the intermediate and native proteins. All the cysteine backbone amide crosspeaks are observed after addition of Fe^{2+} in the presence of DTT.[7] A native-like tertiary structure was also supported by $^{1}H/^{2}H$ exchange data for the intermediate,[8] which demonstrated a similar pattern of slow exchange for most backbone NH protons as previously observed for the holoprotein.[3] Furthermore, it was shown that the native cluster could subsequently be obtained following addition of S^{2-} to a solution of the intermediate,[9] *demonstrating that a relatively stable intermediate could be formed during the initial phase of cluster bio-*

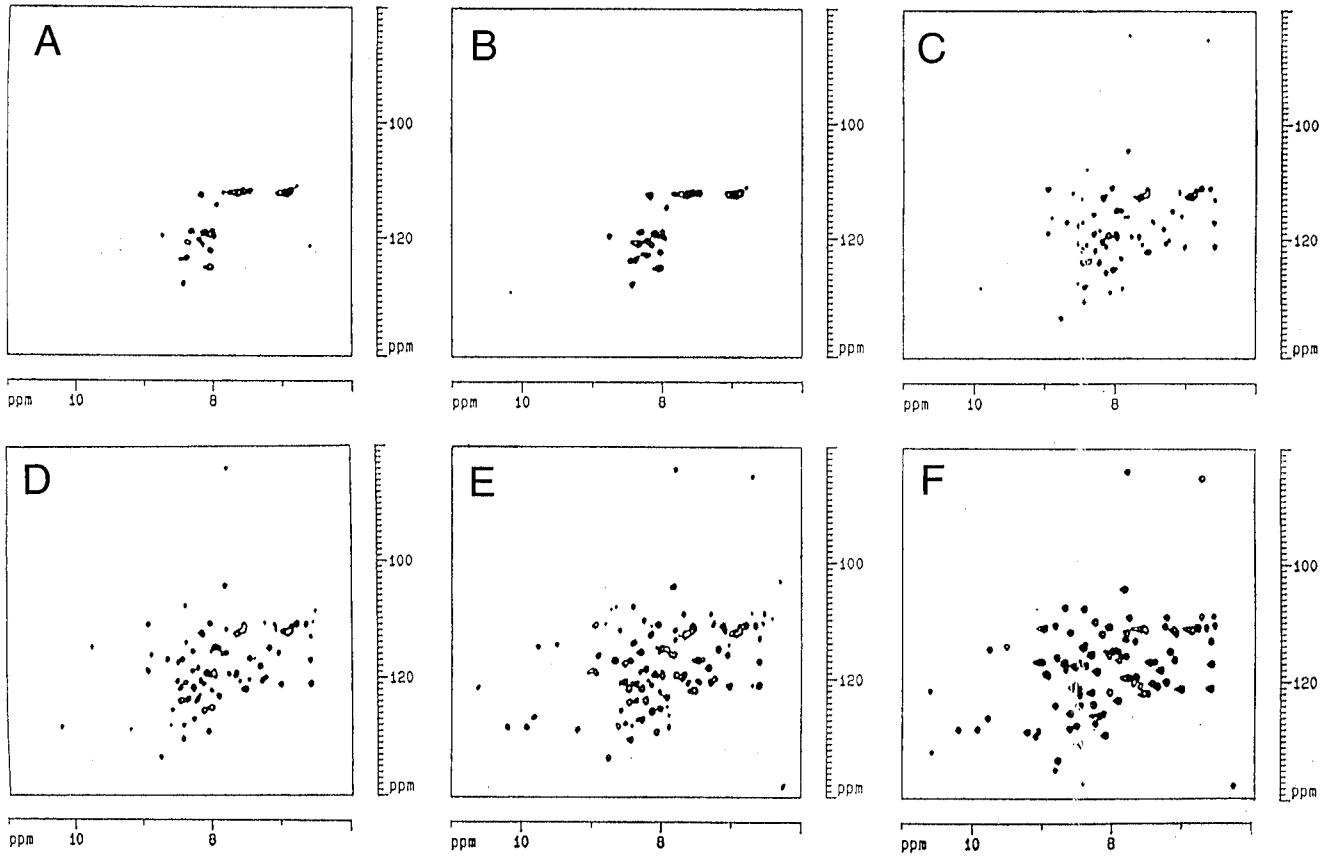

Figure 1. $^{1}H-^{15}N$ NMR Spectra. All NMR experiments were carried out on a Bruker 600 Avance DMX spectrometer operating at a frequency of 600.13000 MHz. The reconstitution experiment was performed using ^{15}N labeled apo-HiPIP (3−5 mM) in 0.1 M Tris buffer at pH 8.0, in 90% H_2O/10% D_2O.[3,14] Apo-protein was incubated with 50 mM DTT for 30 min before the spectral acquisition. After addition of a 16-fold excess of ferrous ion, spectra were accumulated over a period of several hours, each acquisition lasting 10 min. The spectra shown correspond to (A) apo-HiPIP, (B) apo-HiPIP + DTT, (C) apo-HiPIP + Fe^{2+} + DTT (30 min after addition of iron), (D) apo-HiPIP + Fe^{2+} + DTT (2 h after addition of iron), (E) apo-HiPIP + Fe^{2+} + DTT (8 h after addition of iron), and (F) native HiPIP. Two-dimensional spectra were recorded at 298 K by monitoring $^{1}H-^{15}N$ correlation through double INEPT transfer using the pulse program INVIEAGSSI and sensitivity improvement via phase-sensitive echo-antiecho gradient selection.[15−17]

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synthesis. Bacteria have evolved several pathways for the formation and delivery of inorganic sulfide in vivo. These include assimilatory sulfite reduction in *Desulfovibrio* species;[10] *O*-acetylserine sulfhydrylase A and B, and β-cystathionase mobilization of sulfur from cysteine in *E. coli*;[2] and similarly for the NifS in *A. vinelandii*.[1] To our knowledge, the relevant enzymes have not yet been characterized in any *Chromatium* strain.

The iron content of this intermediate was analyzed by the procedure of Moulis and Meyer.[11] Within experimental error, the results indicate the presence of 4 mol of Fe per mol equiv of the intermediate, which is stabilized by the presence of exogenous reducing agents such as dithiothreitol (DTT), and no significant level of sulfide. This stoichiometry is supported by MALDI-TOF mass spectrometry. For the sample conditions employed in the experiment illustrated in Figure 2, the mass difference for the intermediate relative to apo or native HiPIP is consistent with incorporation of 4 equiv of iron and one molecule of DTT. These results were confirmed by experiments with N-15 labeled HiPIP (legend to Figure 2). The DTT presumably complexes to the iron centers to saturate the coordination of each cation. In this regard, glutathione is a biological reductant and source of thiol ligands and is a possible stabilizing agent for cluster assembly in vivo. Additional molecules of bound DTT are too labile to be observed by this method but have been determined by ^{13}C NMR and are required to both saturate the coordination sites of iron centers and stabilize the bound iron centers. This idea is supported by studies of $[Fe_4S_4(SR)_4]$ cluster assembly in the model complexes of Holm and co-workers.[12]

These reconstitution studies indicate that the presence of Fe^{2+} is sufficient to drive cluster formation and backbone folding in HiPIP and that the observed intermediate is most likely a common species in the formation of related biological iron−sulfur clusters. This idea is also consistent with an earlier proposal by Carter, based on a crystallographic analysis of HiPIP,[13] that formation of an initial protein framework is followed by minor rearrangements in torsion angles and the formation of hydrogen bonds to form the native structure. The resulting intermediate most likely contains an Fe-cluster that lacks sulfide bridges but

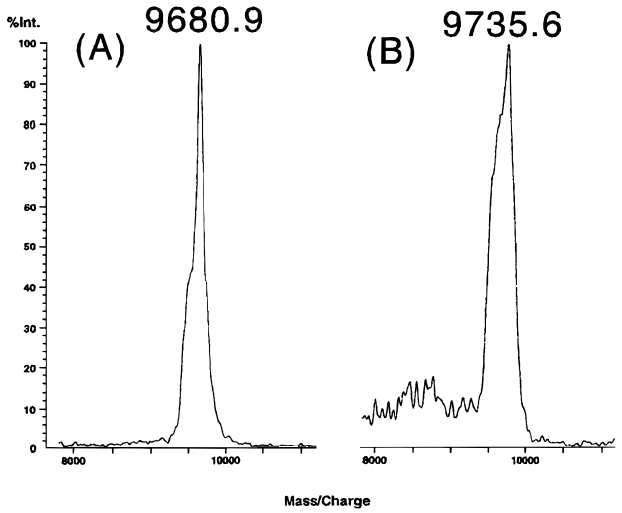
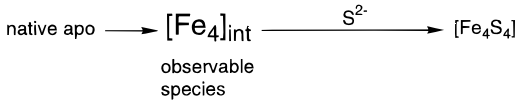


Figure 2. MALDI-TOF Mass Spectrometry. Experiments were run on a Kratos Kompact MALDI III instrument.[18,19] Data obtained for (A) native, and (B) the intermediate, are shown. The intermediate species was prepared as described in refs 5 and 6 (using FPLC purified native HiPIP). A 50 μM solution of the intermediate was dialyzed against 1 mM DTT containing a trace (4 μM) of Fe^{2+}(aq) in order to lower the concentrations of these species to a level where they did not interfere with the experiment. Within the accepted experimental error for the MALDI-TOF technique (0.1−0.3%), the peak observed $\{M + H\}^+$ $[(MW)_{obs} = 9735.6]$ for the intermediate is consistent with the mass $\{M + H\}^+$ of apo-protein and 4 equiv of iron and one of DTT $[(MW)_{calc} = 9723.38]$. Excellent agreement was obtained with results from N-15 labeled HiPIP. The instrument was operated in the linear positive mode at an accelerating potential of 20 kV. The samples were prepared at a concentration of 10 μM in a sinapinic acid (matrix) and 1 μL of solution was loaded onto the sample stage. Horse apomyoglobin was used as an external standard, and the observed molecular weights were based on the calculated average values for the $[M + 2H]^{2+}$ and $[M + H]^+$ ions of horse apomyoglobin.

Scheme 1



is stabilized by ligation to cysteine thiolates from the protein and molecules of DTT. This proposed mechanism for protein cluster assembly (summarized in Scheme 1) is consistent with the fact that native protein could be obtained from the intermediate following addition of S^{2-},[9] although the data currently in hand does not permit an evaluation of the structure of this intermediate cluster for comparison with the synthetic model. Finally, these results demonstrate HiPIP to be an excellent protein model for studies of cluster assembly, and further structural characterization of this intermediate is in progress in our laboratory.

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(5) (a) Apo-HiPIP was prepared from recombinant native [Agarwal, A. et al. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 1357] by following literature procedures [Sola, M. et al., *J. Am. Chem. Soc.* **1989**, *111*, 6627]. Nitrogen-15 labeled apo-HiPIP was prepared from ^{15}N-labeled native protein.[3] (b) Reconstitution was generally carried out by use of the rhodanese method [Pagani, S. et al. *Eur. J. Biochem.* **1984**, *142*, 361] or by direct addition of a 16-fold excess of inorganic sulfide (as Na_2S) as summarized in Sola et al (ref 5a). The protein solution was purged with Ar(g) and incubated with an excess of up to 50 mM dithiothreitol (DTT) prior to the addition of cluster-forming reagents. Ferrous cation (Fe^{2+}) and thiosulfate anion ($S_2O_3^{2-}$) were present in 16-fold excess. Rhodanese was added last.
(6) The Fe^{2+} intermediate of apo-HiPIP was prepared using the same procedure described earlier for reconstitution of native protein[5b] but omitting the addition of $S_2O_3^{2-}$ and rhodanese.
(7) In the case of Cys43 the crosspeak overlaps with a neighboring signal from a Trp ring NH.
(8) The rate of change of the intensities of ^{1}H−^{15}N HSQC cross peaks for a lyophilized sample dissolved in D_2O provides a direct measure of the accessibility of solvent to the backbone amides[3] and consequently the extent of structural ordering. Data were collected as described in the legend to Figure 1.
(9) The ^{1}H NMR, EPR (of the oxidized form), and optical spectra of the protein obtained following addition of inorganic sulfide to a solution of the intermediate were essentially indistinguishable from those of native HiPIP.
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