Conversion of a Fe_2S_2 Ferredoxin into a Ga^{3+} Rubredoxin

Sophia Kazanis,[†] Thomas C. Pochapsky,*[,†]
Terence M. Barnhart,[‡] James E. Penner-Hahn,[‡,§]
Urooj A. Mirza,[||] and Brian T. Chait[||,⊥]

Department of Chemistry and Bioorganic Chemistry Program, Brandeis University Waltham, Massachusetts 02254-9110
Department of Chemistry, The University of Michigan Ann Arbor, Michigan 48109
The Rockefeller University, 1230 York Avenue New York, New York 10021-6399

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The Fe_2S_2 ferredoxin putidaredoxin (Pdx) functions as reductant of cytochrome $P450_{cam}$ in the *Pseudomonas putida* camphor hydroxylase pathway.[1] The structure of oxidized Pdx has recently been elucidated using NMR techniques.[2] However, line broadening due to the unpaired electron spin density renders the nuclei in the vicinity of the Fe_2S_2 cluster unassignable by standard multidimensional NMR methods. It has been shown that rubredoxins, in which a single iron atom is ligated by four cysteinyl sulfurs, can be reconstituted using diamagnetic metals such as Zn^{2+}, Cd^{2+}, and Hg^{2+}, thereby allowing sequential resonance assignment of residues in the vicinity of the metal binding site.[3−5] It has also been shown that Fe_2S_2 ferredoxins can be reconstituted *in vitro* from the apoferredoxin upon addition of iron and sulfide.[6−8] Short-lived species apparently containing non-native iron coordination have also been observed.[9,10] Recently, exchange of Cd^{2+} for iron in Fe_4S_4 centers has been reported, although the nature of the Cd^{2+} incorporation is as yet unclear.[11] Here, we report that reconstitution of Pdx using Ga^{3+} yields a stable mononuclear rubredoxin-like (GaS_4) gallium derivative of Pdx (GaPdx) which retains the secondary structure and global fold of native Pdx.

GaPdx was prepared using the reconstitution procedure of Tsibris *et al.* except that $FeCl_3$ was replaced with $GaCl_3$.[8,12]

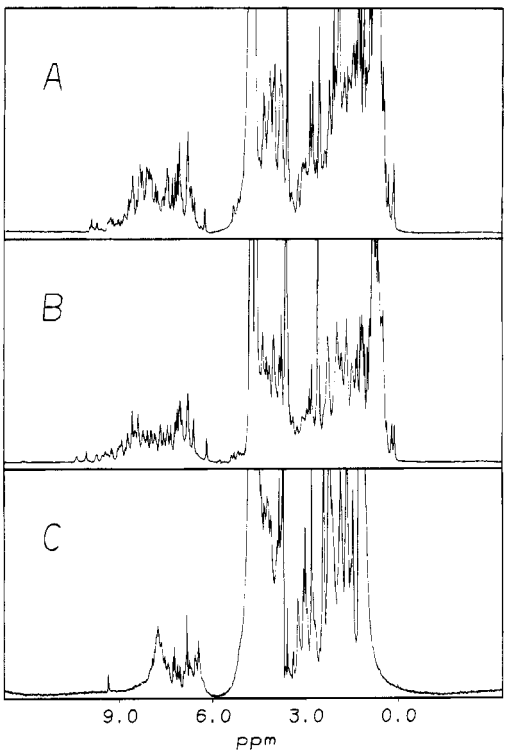

Figure 1. Comparison of 500 MHz ^1H NMR spectra of (A) GaPdx, (B) native (Fe_2S_2) oxidized Pdx, and (C) apo-Pdx (no metal). Samples were 3−4 mM protein in 90% H_2O/10% D_2O containing 50 mM deuterated Tris HCl (pH 7.4) and 10 mM 2-mercaptoethanol.

The reconstitution results in better than 90% yield of GaPdx, as estimated by comparison of ^1H NMR signal intensities before and after reconstitution. The NMR spectrum of GaPdx is very similar to that of native Pdx, unlike apo-Pdx, which exhibits the spectrum of an unfolded protein (Figure 1). Substitution of $Ga(NO_3)_3$ for $GaCl_3$ yields a spectrally identical reconstitution product. Even if sulfide ion is present during reconstitution, the reconstituted protein does not incorporate sulfide, based on a colorimetric assay.[13] Samples of native Pdx and GaPdx were compared by negative ion electrospray mass spectrometry (MS); native Pdx gave a measured molecular mass of 11 591 ± 5 daltons (Da) and GaPdx a measured molecular mass of 11 482 ± 4 Da. Positive ion electrospray MS of acid-denatured forms of both native Pdx and GaPdx gave a measured mass of 11 415 ± 4 Da for the apoprotein in both cases, which is in agreement with the value calculated from the known amino acid sequence (11 419 Da).[14] These results indicate that GaPdx incorporates a single gallium atom. The calculated molecular mass of native Pdx is 11 591 Da, and that of GaPdx is 11 485 Da.

The EXAFS (extended X-ray absorption fine structure) spectrum of GaPdx is consistent with Ga coordination by four sulfur atoms with an average Ga−S bond distance of 2.27 ± 0.02 Å with a Debye−Waller factor (σ^2) of 3.8×10^{-3} Å^2.[15] Although the involvement of N or O ligands in Ga coordination cannot be definitively excluded by EXAFS, the data is extremely well fitted using only S_4 ligation (Figure 2). Differences

[†] Brandeis University.
[‡] The University of Michigan.
[§] Inquiries regarding EXAFS analysis should be directed to this author.
[||] The Rockefeller University.
[⊥] Inquiries regarding mass spectral analysis should be directed to this author.

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(12) Native Pdx was diluted to 1 mg/mL with argon-saturated 50 mM Tris HCl buffer pH 7.4 containing 50 mM mercaptoethanol. 100% trichloroacetic acid was added to 20% v/v. The precipitated apoprotein was collected by centrifugation, and the pellet was washed 3× with cold degassed 20% trichloroacetic acid. The precipitate was redissolved in 10 mL of 0.1 M Tris (pH 9.2) containing 50 mM mercaptoethanol, and a 6-fold molar excess of aqueous $GaCl_3$ solution was added. The product solution was concentrated and purified by gel filtration chromatography.

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(14) Electrospray ionization mass spectra were obtained with a Finnigan-MAT TSQ-700 triple-quadrupole mass spectrometer. Samples of native Pdx and GaPdx were electrosprayed at a concentration of 50 μM from pure water solutions, whereas the denatured form of the proteins was electrosprayed at a concentration of 20 μM from water/methanol/acetic acid (45%/50%/5% v/v/v). The analyte solutions were infused into the mass spectrometer source at a rate of 3 μL/min through a 100 μm i.d. fused silica capillary. The mass spectra were obtained by averaging signals over a period of 1 min. Molecular masses were calculated using the published sequence obtained by nucleotide sequencing (Peterson, J. A.; Lorence, M. C.; Amarneh, B. *J. Biol. Chem.* **1990**, *265*, 6066−6073).

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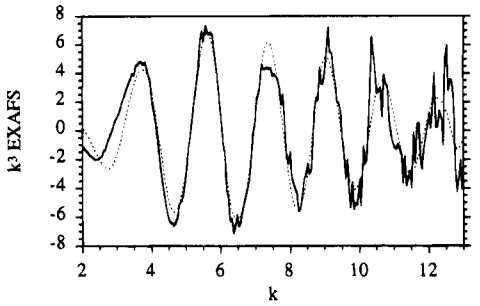

Figure 2. k^3-weighted EXAFS spectrum of GaPdx (solid line) compared to a theoretical fit (dashed line) using FEFF 6.1 for an S_4 first-shell ligation of Ga^{3+}.

between the X-ray near edge spectra (XANES) of GaPdx and $Ga(SR)_4^-$ model compounds in which the Ga is tetrahedrally ligated suggest a distortion of tetrahedral geometry toward square planar. As the cysteinyl sulfur atoms ligating the Fe_2S_2 center in the native protein are likely to be coplanar, such a distortion would not be surprising.

GaPdx is sufficiently stable at ambient temperature and neutral pH to be characterized by standard NMR methods. Sequential ^1H and ^{15}N resonance assignments are similar to those of native Pdx (Figure 3).[2,16] It is also evident from NOE connectivities that the major secondary structural features and global fold of native Pdx are conserved in GaPdx. Since GaPdx is diamagnetic, many resonances which could not be assigned in the native protein have already been assigned in the spectrum of GaPdx. These include the ^1H and ^{15}N resonances for residues Leu 84-Cys 85-Cys 86-Gln 87, none of which could be assigned in the native protein, as Cys 86 (along with Cys 39, Cys 45, and Cys 48) provides one of the thiolate ligands for the Fe_2S_2 cluster. Also assigned are the previously unobserved side chains of Met 24, Met 70, and Leu 71, all of which are near the metal cluster in native Pdx. Resonances in the metal cluster binding loop (Val 36–Cys 48), including Gly 37, Gly 40, and Gly 41, have also been assigned in GaPdx. Complete assignment of the ^1H, ^{13}C, and ^{15}N spectra of GaPdx, as well as a complete tertiary structural refinement, is in progress.

Since all of the identified structural features of native Pdx are conserved in GaPdx, we conclude that the only significant structural differences between native Pdx and GaPdx will be found in the flexible metal binding loop (residues 36–48), which is apparently sufficiently distortable to accommodate a range of metal binding geometries. This raises some fascinating questions, including why a Fe_2S_2 cluster is selected for exclusively *in vivo*, and what role the cluster might play in ferredoxin folding. Preliminary amide proton exchange measurements indicate that GaPdx is more dynamic than the native protein, with significantly increased amide proton exchange rates relative to the native protein (although the protons which show slow exchange in the native protein are also the slowest exchanging in GaPdx (S. Kazanis, unpublished results)). It is tempting to speculate on the basis of these observations that GaPdx may represent a trapped late intermediate in the Pdx folding pathway.

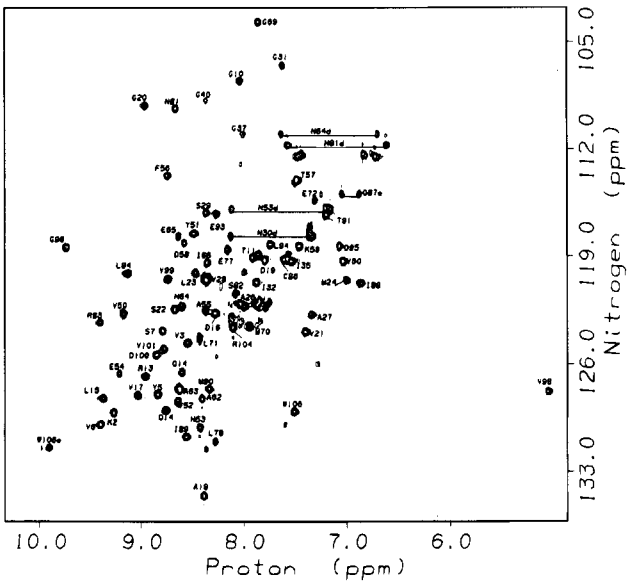

Figure 3. 500 MHz $^1H-^{15}N$ HSQC (heteronuclear single-quantum correlation) spectrum of uniformly ^{15}N-labeled GaPdx showing correlations between directly bonded ^{15}N and ^1H nuclei. Numbers correspond to amino acid sequence assignments, as determined from two-dimensional ^1H NOESY, 2QF-COSY, 2Q, TOCSY, and three-dimensional $^1H-^{15}N$ NOESY-HSQC and $^1H-^{15}N$ TOCSY-HSQC spectra. Samples were 3–4 mM protein in 90% H_2O/10% D_2O containing 50 mM deuterated Tris HCl (pH 7.4) and 10 mM 2-mercaptoethanol.

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(15) X-ray absorption spectra were measured in the fluorescence mode at the Stanford Synchrotron Radiation Laboratory (SSRL), beamline 7-3. Samples were held at 10K throughout data acquisition. Data were analyzed using standard procedures and fitted using *ab initio* models calculated with FEFF 6.1 (see: Rehr, J. J.; Zabinsky, S. I.; Albers, R. C. *Phys. Rev. Lett.* **1992**, *69*, 3397. Rehr, J. J.; Mustre de Leon, J.; Zabinsky, S. I.; Albers, R. C. *J. Am. Chem. Soc.* **1991**, *113*, 5135–5151). The amplitude and phase parameters were calibrated by fitting data for compounds of known structure. Unfiltered k^3-weighted data were fitted by allowing R_{Ga-S} and σ^2 factor to vary. The apparent coordination number was fixed and systematically increased in units of 0.5 to find the best fit. Monochromator energy was calibrated by assigning the first inflection point of the GaPdx absorption edge as 10 367 eV.

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