Probing Copper−Thioether Coordination Chemistry in Rusticyanin and Azurin by 2D $^{1}H-^{199}Hg$ NMR

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Many spectroscopic methods are useful for probing Cu(II)−thiolate and −imidazole coordination in proteins, but few can provide insights into Cu(II)−thioether interaction.[1] The prospects are worse for d^{10} centers in the emerging class of copper-detoxification and transport proteins where cysteine and methionine are potential Cu(I) ligands.[2] We are developing mercury-based methods to evaluate the inorganic chemistry of copper proteins and have shown that the ^{199}Hg chemical shift of mercury substituted into the type 1 copper site of plastocyanin is deshielded by 135 ppm relative to azurin, consistent with a weaker Hg−thioether interaction in the latter.[3] Mercury−proton correlation experiments provide more detailed insights into the coordination environment in copper proteins.[4] Using these methods, we report here that Hg(II) faithfully reflects subtle differences in the copper−thioether interactions in azurin and plastocyanin, where the Cu−S(Met) distances are reported as 3.12 and 2.82 Å, respectively.[5−7] These methods also reveal that the ligand environment of the high-potential blue copper protein rusticyanin is best described as $His_2CysMet$. We conclude that Hg−rusticyanin, and by inference, the Cu−protein, has a distorted tetrahedral coordination site more similar to plastocyanin than azurin. Since Hg(II) readily adopts the ligands imposed by these proteins on the native metal, it is likely that these ^{199}Hg NMR methods will be useful probes of subtle aspects of the coordination chemistry in other copper proteins.

Rusticyanin, a principal component of the electron transport chain of the iron-oxidizing bacterium *Thiobacillus ferrooxidans,* exhibits an unusually high redox potential and atypical acid stability.[8,9] The issue of how the coordination environment in rusticyanin differs from well-characterized type 1 centers such as azurin and plastocyanin has been controversial. Proposed copper ligands in rusticyanin include a histidine nitrogen, a cysteine thiolate, and a methionine thioether,[10−16] with aspartate carboxylates, carbonyl oxygens, or histidine nitrogens suggested as potential fourth or fifth ligands.[11,12] Recent ^{1}H NMR and EXAFS studies support the role of His85 as the fourth ligand.[17−19]

Mercury derivatives of rusticyanin and azurin were prepared by the addition of several equivalents of ^{199}HgO to the apoproteins[3,20] and final analysis revealed a metal-to-protein mole ratio of 0.8 in both cases. The 2D Fourier transform $^{1}H\{^{199}Hg\}$ HMQC NMR spectra were obtained with a Bruker 600 spectrometer (14.09 T)[4] and are shown in Figure 1.

The proton-detected HMQC spectrum of ^{199}Hg−azurin reveals signals from three amino acid side chains that are ligated to the ^{199}Hg center exhibiting a chemical shift of −880 ppm. The ^{1}H NMR resonance at 2.95 ppm is assigned to the cysteine C_β-$^{1}H_2$ and resonances at 7.17, 7.09, 7.02, and 6.21 ppm are assigned to protons of two histidine rings. No evidence for other ligands to the mercury was obtained using delay (Δ) values from 1.5 to 20 ms. In ^{199}Hg−plastocyanin a fourth ligand was clearly identified as methionine in both X-ray crystallography[21] and in $^{1}H\{^{199}Hg\}$ HMQC NMR experiments.[4] No ^{199}Hg coupling with ^{1}H spins of methionine is observed in Hg−azurin, consistent with little if any σ-bonding character in the Hg−S(Met) bond. Enhanced methyl T2 relaxation arising from dynamic features of a weak Hg−S(Met) interaction in azurin could also diminish the correlation signal. In contrast to Hg−azurin, appreciable Hg−S(Met) σ character is clearly present in plastocyanin. This conclusion correlates well with structural data for the copper proteins: Cu−S(Met) distances are typically much longer in azurins (3.12 Å for *P. aeruginosa*)[5] than plastocyanins (2.82 Å for poplar plastocyanin).[6,7] High-resolution structural data are not available for copper−ligand distances in rusticyanin.

These HMQC results support an earlier interpretation of the ^{199}Hg chemical shift differences between Hg−plastocyanin (−749 ppm) and Hg−azurin (−884 ppm) wherein the additional shielding in the latter is ascribed to weakening or loss of the methionine−Hg(II) interaction and/or additional Hg−carbonyl oxygen interaction.[3] We cannot rule out the possibility that metal interaction with the carbonyl oxygen of Gly45 in azurin contributes to the shielding difference, since such a displacement

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(20) Aporusticyanin was prepared as previously described.[26] Isotopically enriched ^{199}HgO (91.1%, purchased from Oak Ridge National Laboratories) was dissolved in 6 M acetic acid and diluted with Tris-HCl buffer (50 mM, pH 7.8). The Hg(II) solution was added to apoprotein in Tris-HCl buffer and allowed to react for 4 days at room temperature. Excess mercuric ion was removed by gel filtration using Sephadex G25. Hg(II)−rusticyanin was exchanged into 60 mM sodium acetate in D_2O, pH 5.5, and concentrated using Centricon-10 concentration devices.
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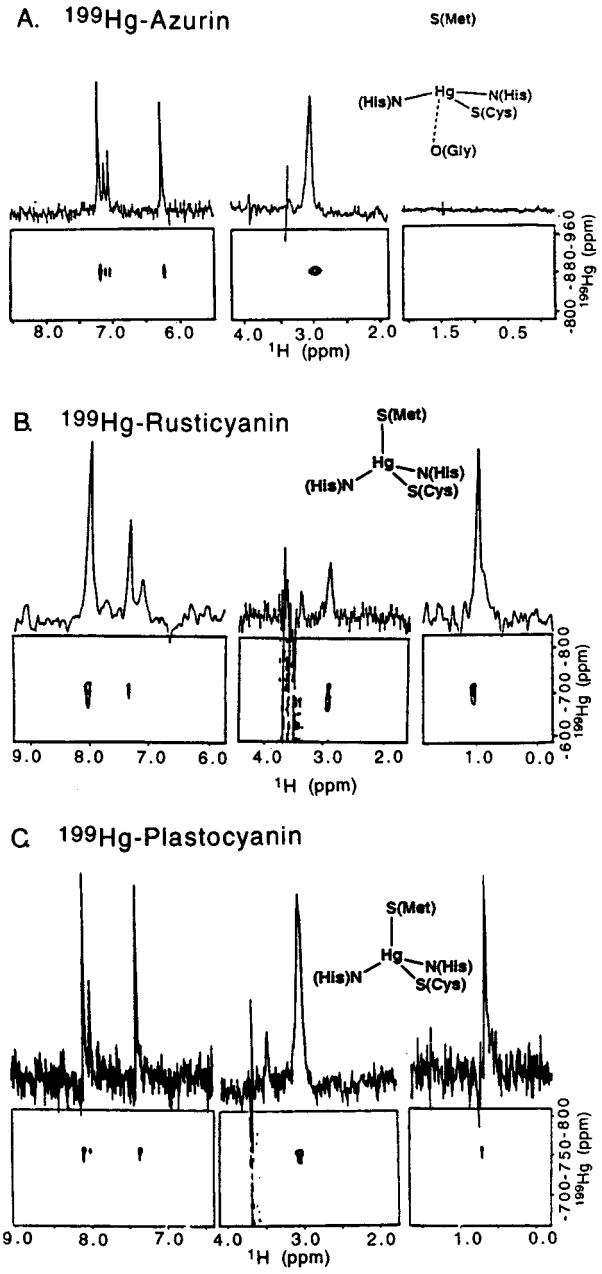

Figure 1. $^1H\{^{199}Hg\}$ HMQC NMR spectra of mercury-substituted azurin (*P. aeruginosa*) and rusticyanin (*T. ferrooxidans*) obtained with a Bruker 600 spectrometer (14.09 T, 107.4 MHz for ^{199}Hg) using the pulse sequence $\pi/2_x(^1H)-\Delta-\pi/2_\phi(^{199}Hg)-t_1/2-\pi_x(^1H)-t_1/2-\pi/2_x(^{199}Hg)-\Delta-Acq\ (^1H)$ (decouple ^{199}Hg),[25] as previously described.[4] (A) The spectra were acquired on a 6.2 mM ^{199}Hg−azurin solution in 4 mM ammonium acetate, pH 8.5, 97% (v/v) D_2O, and those in (B), on a 0.5 mM ^{199}Hg−rusticyanin sample in a 98% D_2O buffer, 60 mM sodium acetate, pH 5.5. ^1H slices through the corresponding ^{199}Hg shift are shown at the top of each spectral region displayed. A preparation delay $\Delta = 15$ ms was used for the first and third spectral regions, while $\Delta = 3$ ms was used for the middle regions displayed for each protein. The ^{199}Hg−azurin spectra were collected with a spectral width of 7246 Hz for ^1H (F2) and 42 960 Hz for ^{199}Hg (F1). A total of 256 transients were collected in F2 for each of 64 t_1 blocks that were accumulated. Total acquisition times were 6 h for each spectrum. The ^{199}Hg−rusticyanin spectra were collected with a spectral width of 7246 Hz for ^1H (F2) and 42 967 Hz for ^{199}Hg (F1). A total of 840 transients were collected in F2 for each of 64 t_1 blocks that were accumulated. Total acquisition times were 21 h for each spectrum. The ^{199}Hg−plastocyanin spectra are shown for comparison.[4] All spectra were ^{199}Hg-decoupled with a GARP decoupling scheme, and the residual HOD signal was selectively presaturated during the relaxation delay.

was observed for Cd−azurin.[22] Intriguingly, the energetically favorable formation of a Hg−thioether bond is not sufficient to overcome the forces stabilizing the tertiary fold of azurin. Having established that mercuric ion generally adopts the primary coordination environment imposed by these two proteins on the native copper atom, we extend the methods to a site of unknown structure in rusticyanin.

The 2D HMQC spectra of ^{199}Hg−rusticyanin reveal methyl ^1H peaks correlating with the ^{199}Hg resonance at −706 ppm. This ^1H resonance at 0.98 ppm (Figure 1B) is assigned to methionine Cϵ-$^1H_3$. Three distinct histidine ring protons at 7.99, 7.29, and 7.08 ppm indicate ligation to the mercury center by two histidine residues. As with ^{199}Hg−plastocyanin, a fourth histidine proton is not observed.[4] The ^1H peak at 2.89 ppm falls within the methylene region and is assigned to cysteine C$_\beta$-$^1H_2$. This peak was observed only at the shorter delay of 3 ms, corresponding to a large coupling constant expected for cysteine coordination.[4] The lower intensity peak at 3.36 ppm is also observed for Hg−plastocyanin[4] and is attributed to either a Cys C$_\beta$-$^1H_2$ or a Met Cγ-$^1H_2$.

The rusticyanin $^1H\{^{199}Hg\}$ HMQC spectrum is most similar to that observed for plastocyanin.[4] The presence of Met Cϵ-$^1H_3$ resonances in these $^1H\{^{199}Hg\}$ HMQC spectra is consistent with a $^3J_{H-Hg}$ coupling path that is dependent on σ bonding and suggests significant covalent character in the Hg−S(Met) interaction for both rusticyanin and plastocyanin. A weak but discernible bonding interaction between the copper and methionine thioether is also apparent in electronic structure analysis of Cu−plastocyanin.[1,23,24] On the other hand, an Hg−O(Gly) or Hg−O(Asp) interaction would be difficult to observe in $^1H\{^{199}Hg\}$ HMQC spectra because the ^4J coupling should be weak. Similar interactions have not yet been detected using these ^{199}Hg NMR methods.

The ^{199}Hg chemical shift provides further insights into the coordination environment of rusticyanin. Direct-observe ^{199}Hg NMR experiments confirm a single ^{199}Hg resonance at −706 ppm (line width of 1355 Hz; spectrum not shown) for Hg−rusticyanin. In comparison to the 1000 ppm range observed for ^{199}Hg−protein chemical shifts, the −706 ppm value for rusticyanin is close to that of plastocyanin (−749 ppm) and is most consistent with a $His_2CysMet$ environment.[3] Other possibilities such as the coordination of a metal−carboxylate proposed by Blake and co-workers[11] or interaction of a carbonyl oxygen, such as the fifth ligand observed in Cu−azurin, are expected to shield, not deshield, the Hg(II) nucleus[3] and therefore are ruled out. The 43 ppm deshielding of rusticyanin relative to plastocyanin is consistent with a stronger interaction between Hg(II) and the sulfur ligands in the former; however, additional data on model compounds will be useful since ^{199}Hg chemical shifts can be influenced by a variety of electronic and geometric factors.

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