Structural Characterization of Mercury-Substituted Copper Proteins. Results from X-ray Absorption Spectroscopy

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Abstract: Extended X-ray absorption fine structure (EXAFS) and X-ray absorption near-edge structure (XANES) data are reported for Hg-substituted derivatives of the blue copper proteins plastocyanin, azurin, tree laccase, and stellacyanin. Analysis of EXAFS data confirms that the Hg is selectively substituted at the type 1 (blue) site in laccase and provides the first direct structural information about this metal-binding site. In every case the primary ligands coordinated to mercury are two imidazole groups and one thiolate sulfur derived from the side chains of histidine and cysteine residues, respectively. Together with crystallographic results available for Hg plastocyanin, the data reveal that the coordination geometry of the type 1 site (but not the metal–ligand bond distances) is essentially the same whether copper or mercury is bound. Reasons why the preparation of the mixed-metal derivative of laccase can be achieved are discussed, and this technique is established as a viable means for the chemical modification of enzymes containing multiple copper centers. The Hg XANES data provide clear evidence for a structural difference in stellacyanin compared with the other three proteins. These results and the second-shell EXAFS data are interpreted in terms of a difference in the distal ligand(s); however, we have not been able to identify the group(s) involved.

The type 1 (or blue) copper center is found in a number of proteins involved in biological redox processes. Distinctive chemical and physical properties of the site include[1-3] (1) intense visible absorption at around 600 nm (ϵ = 4000–5000 M^{-1} cm^{-1}), (2) narrow hyperfine splitting in the $g_\parallel$ region of the EPR spectrum [(40–80) × 10^{-4} cm^{-1}], and (3) a relatively positive reduction potential (200–800 mV vs SHE). By now, the basic structural properties of the blue copper site are fairly well understood. X-ray crystallography[4,5] has revealed that the immediate coordination environment of the type 1 copper in plastocyanin consists of a roughly trigonal arrangement of two imidazole nitrogens from histidine side chains and one thiolate sulfur from cysteine. A methionine sulfur that is 2.9 Å from the copper completes the coordination sphere. The methionine is evidently very weakly bound as it appears to make no appreciable contribution to the copper EXAFS even at 4 K.[6,7] However, the methionine sulfur evidently has some influence on the energy of the d_{z^2} orbital and hence on the ligand field absorption bands,[8] as would be expected from the fact that the Cu–S(Met) bond is nearly perpendicular to the $d_{x^2-y^2}$ orbital, which contains the unpaired electron.[9] In the blue copper proteins that have been structurally characterized to date, the same three equatorial ligands are always observed, but the nature of the axial ligation varies.[4,5,10-12] For example, in cupredoxin from S-6 *Alcaligenes faecalis*[12] the methionine sulfur is closer to the copper center (2.69 Å) than it is in plastocyanin whereas in azurin there is a peptide oxygen, opposite the methionine, which might be considered to function as a distant, fifth ligand.[10,11] Still different ligation must occur in stellacyanin since the protein contains no methionine residues.[13]

Type 1 copper is also found in the blue oxidases, such as laccase, which couple the oxidation of various substrates to the four-electron reduction of dioxygen to water.[2] Kinetic studies indicate that at least three of the four electrons required for dioxygen reduction are taken up via the type 1 site of laccase.[14-16] Two other spectroscopically distinct sites, the type 2 and the type 3 copper sites, apparently function together in the reduction of the dioxygen substrate.[17-19] Unfortunately, spectroscopic signals from the type 2 and the type 3 sites are complicated by overlap with those of the type 1 copper.[2] In an attempt to circumvent this problem, Morie-Bebel et al. showed that reconstitution of the type 1 site could be blocked by treating apolaccase with Hg(II).[20] When copper is subsequently incorporated into the remaining sites, the spectral signals of type 2 and type 3 copper are resolved in much greater detail.[21,22] The mercury derivative of laccase could be useful in a variety of other studies; for example, reoxidation of the reduced protein could provide an entry into the three-electron-reduced dioxygen intermediate that has been observed in studies of the native enzyme.[14] The preparation of analogous, mixed-metal derivatives could also be useful in studies of even more complicated enzymes such as ceruloplasmin and ascorbate oxidase.

Because of the continuing interest in this modification, we have investigated the mercury derivative of laccase by Hg X-ray absorption spectroscopy in order to identify the locus of mercury

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binding. For comparison, we have also examined the mercury derivatives of the smaller blue copper proteins azurin, plastocyanin, and stellacyanin. Hg plastocyanin serves as an important control since Freeman and co-workers have recently solved its crystal structure.[23]

Materials and Methods

Acetone powder of the latex of the Chinese lacquer tree *Rhus vernicifera* was obtained from Saito and Co., Ltd. (Osaka, Japan). Laccase and stellacyanin were extracted by the method of Reinhammar[24] with minor modifications. The bacterial culture of *Pseudomonas aeruginosa* (strain 10145) was obtained from the American Type Culture Collection (Rockville, MD). The bacteria were cultured, and azurin was extracted as previously described.[25,26] Plastocyanin was isolated from spinach leaves and purified as described by Markley et al.[27] All chemicals were of reagent grade and were used as obtained. Deoxygenated buffer solutions were prepared by purging with N_2(g) that had been passed through solutions containing vanadous ion over zinc amalgam. Buffers were prepared from deionized water and passed through a Chelex-100 (Bio-Rad) column to remove trace metal contaminants. The mercury derivative of laccase was prepared as before.[20] The Hg(II) derivatives of the other proteins were prepared by the addition of 1–2 equiv of Hg(II) to the apoprotein followed by dialysis to remove excess metal. In all cases the mercury to protein stoichiometry was 1.0 ± 0.1. Apoplastocyanin was prepared following the procedure outlined by McMillin,[28] while apoazurin and apostellacyanin were prepared by the method of Blaszak et al.[29] One sample of apostellacyanin was also prepared by dialysis against cyanide,[30] and there was no difference in the spectral properties of the resulting mercury derivative. For the XAS (X-ray absorption spectroscopy) studies the protein concentrations were ca. 2 mM in 0.1 M pH 6 phosphate buffer, and the samples were maintained and studied at −70 °C in Lucite cells with Mylar windows.

X-ray Absorption Measurements. The XAS data were measured at the Stanford Synchrotron Radiation Laboratory (SSRL) with the synchrotron radiation at beam line VII-3, under dedicated conditions (3.0 GeV, 40–50 mA). Data were measured as fluorescence excitation spectra, with either an array of NaI(Tl) scintillators or a single large-solid-angle ion chamber to detect the X-ray fluorescence. The X-ray beam size was 2 × 12 mm, and the incident beam was monochromated with the (220) reflection of a silicon crystal in a double-crystal monochromator. Incident intensity was monitored with a N_2-filled ionization chamber. A second and a third ionization chamber, placed behind the sample, were used to measure the absorption of Hg/Sn amalgam simultaneously with data collection such that there was an independent energy calibration for each scan. The first inflection point of the Hg L_3 edge of the amalgam was assigned an energy of 12 284.0 eV.[31] Spectra were measured for 700 eV above the L_3 absorption edge, corresponding to k_{max} = 13. The first and last scans for each sample were compared to check for the possibility of radiation damage. No detectable change was observed for any sample. Two samples each were measured for Hg laccase and Hg stellacyanin. Three samples were measured for Hg plastocyanin, and one sample was measured for Hg azurin. In no case were sample-dependent structural variations observed.

Data Analysis. The background was removed by fitting a polynomial to the preedge region and subtracting the extrapolated polynomial from the entire data set. The EXAFS amplitude χ was defined as $\chi = (\mu - \mu_s)/\mu_o$, where μ is the preedge-subtracted absorption, μ_o is the Victoreen fall off in the absorption above the edge,[32] and μ_s is a polynomial spline fitted to the absorption above the edge.

The data were converted to k space according to $k = [8\pi^2 m_e(E - E_o)/h^2]^{1/2}$, where m_e is the electron mass and E_o was set to 12300.0 eV. All Fourier transforms and curve fits were performed with k^3-weighted data. Spline points were chosen so as to give the maximum amplitude for the first-shell peak and the minimum amplitude for $R < 1$ Å in the Fourier transform. Fourier filtering was used to isolate the different shells of EXAFS data, with the first shell taken as R = 1.2–2.3 Å and the second shell as R = 2.3–3.3 Å. In addition, we calculated a wide filter including both shells over the range R = 1.0–3.3 Å. Fits of the wide-filter data are provided as supplementary material.

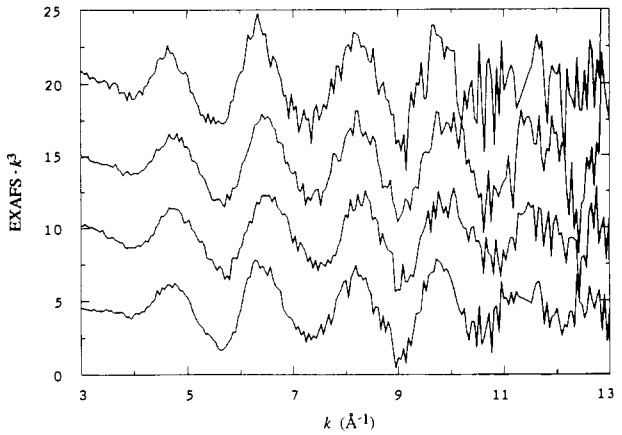

Figure 1. EXAFS spectra for Hg-substituted blue copper proteins. From top: azurin, plastocyanin, laccase, and stellacyanin. Data are weighted by k^3 to enhance the visibility of the oscillations at high k. Spectra have been displaced vertically for enhanced visibility.

The EXAFS data were fitted in a nonlinear least-squares sense to the model

$$\chi(k) = (N/R^2)A(k)\exp(-2k^2\sigma^2)\sin[2kR + \phi(k)] \quad (1)$$

where N is the number of scatterers at a distance R and σ^2 is the mean square deviation in R. The amplitude, $A(k)$, and phase, $\phi(k)$, parameters were determined empirically from measured EXAFS data for the model compounds: $[Hg(pyridine)_2](ClO_4)_2$ (Hg–N and Hg···C parameters) and $Hg(SCN)_2$ (Hg–S parameters).[33] In all cases, the coordination numbers were fixed at integer values, and only the absorber–scatterer distances and the Debye–Waller-like factors [$\exp(-2k^2\sigma^2)$] were varied. Equivalent results (not shown) were obtained in fits using ab initio parameters,[34] calibrated by comparison with model compounds.[35]

For the first shell, best fits of the filtered data were obtained with either S only, N only, or a combination of S and N atom scatterers. In all cases, the best fit corresponded to two nitrogens and one sulfur. We expected the second-shell data to contain contributions from imidazole carbons, possibly the methionine sulfur, and, at least for azurin, a peptide oxygen. The data were accordingly modeled by 4C only, by 1S only, and by combinations of 4C with either S or O. (Hg–N parameters were used in lieu of an authentic Hg–O model.) With the wide filter of range 1.0–3.3 Å, several different combinations of 2N, S, 4C, S′, and N′ were used. In all cases results obtained with the wide or the narrow filter were equivalent. We estimated the uncertainties in each bond length R by determining the variation, ΔR, required to increase the mean derivation between data and fit by a factor of 2. From this we estimate the uncertainty as ±0.03 Å for the first shell and ±0.06 Å for the second shell. The uncertainty in $\Delta\sigma$ is estimated as ca. ±0.02 Å. As expected, there is a weak negative correlation between the first-shell bond lengths.

The data reduction described above does not in general result in accurately normalized spectra in the immediate vicinity of the absorption edge (the X-ray absorption near structure or XANES region). We have developed a new procedure[36] for normalizing XANES spectra in which a low-order polynomial is subtracted from the data and the resultant is multiplied by a scale factor. The polynomial and scale factor are chosen to give the maximum agreement, both below and well above the edge, with tabulated X-ray absorption cross sections.[31]

Results and Discussion

The k^3-weighted EXAFS of the four Hg-substituted proteins are shown in Figure 1 and their corresponding Fourier transforms in Figure 2. Since the EXAFS spectra of the four proteins are all very similar and since mercury is known to bind at the type

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Table I. Best Fits of Models for the First-Shell Data

protein	CN[a]	R (Å)	$\Delta\sigma$ (Å)[b]	CN[a]	R (Å)	$\Delta\sigma$ (Å)[b]	F[c]
Hg plastocyanin	3N	2.24	−0.011				0.50
	2S	2.34	−0.007				0.67
	2N	2.25	−0.013	1S	2.32	+0.002	0.16
Hg azurin	4N	2.25	+0.008				0.43
	3S	2.37	+0.015				0.50
	2N	2.24	+0.001	1S	2.38	0.000	0.13
Hg laccase	3N	2.22	−0.002				0.54
	2S	2.33	−0.001				0.40
	2N	2.24	+0.012	1S	2.32	−0.009	0.14
Hg stellacyanin	4N	2.25	+0.008				0.49
	2S	2.36	+0.001				0.43
	2N	2.24	+0.006	1S	2.37	−0.006	0.07

[a] Number of atoms of the indicated type in the fit. Coordination numbers were fixed at various integer values. The coordination numbers listed here are those which gave the best fit to the data. [b] Variation in σ relative to model compound. [c] Goodness of fit indicator defined as $F = [\sum(\chi - \chi_{calc})^2 k^6]^{0.5}/(N-1)$.

Table II. Best Fits of Models for the Filtered Second-Shell Data

protein	CN[a]	R (Å)	$\Delta\sigma$ (Å)[b]	CN[a]	R (Å)	$\Delta\sigma$ (Å)[b]	F[c]
Hg plastocyanin	4C	3.25	+0.027				0.15
	1S	2.96	+0.038				0.13
	4C	3.22	+0.041	1S	2.98	+0.051	0.11
	4C	3.26	+0.030	1O	2.80	+0.032	0.094
Hg azurin	3C	3.27	−0.030				0.20
	1S	3.02	−0.011				0.26
	4C	3.26	−0.019	1S	3.09	+0.031	0.12
	4C	3.27	−0.017	2O	2.91	+0.051	0.12
Hg laccase	3C	3.29	+0.007				0.15
	1S	3.01	+0.029				0.17
	4C	3.27	+0.017	1S	3.08	+0.064	0.12
	4C	3.28	+0.018	2O	2.90	+0.070	0.11
Hg stellacyanin	4C	3.26	0.000				0.16
	1S	3.00	+0.006				0.15
	4C	3.24	+0.006	1S	3.04	+0.034	0.08
	4C	3.26	+0.006	1O	2.89	+0.017	0.067

[a] Number of atoms of the indicated type in the fit. [b] Variation in σ relative to model compound. [c] Goodness of fit indicator defined as in Table I.

1 site in plastocyanin,[23] the most evident conclusion is that Hg binds at the type 1 site in all four proteins. This is an important finding because it provides the first *direct* evidence that mercury selectively replaces the type 1 copper in laccase.

Quantitative analyses of the first-shell data are presented in Table I. These confirm the expected structure[23] and demonstrate that the first coordination sphere ligands bind at virtually identical distances in all four proteins. Distinctly better fits are obtained with a three-coordinate 2N + S shell than with a nitrogen-only or a sulfur-only shell. In the crystal structure determination of Hg plastocyanin, Church et al. have reported a quite short Hg–S(Cys) bond of 2.38 Å, a long Hg–S(Met) interaction at 3.02 Å, and "normal" Hg–N(His) bonds with lengths of 2.34 and 2.36 Å, respectively.[23] The differences in the bond distances obtained by EXAFS are not large in comparison with experimental error; they agree to within two standard deviations. If the differences are real, they might be explained by differences in the method of preparation. For the crystallographic work the mercury derivative was apparently prepared by soaking a crystal of copper plastocyanin in a solution containing Hg(II). Conceivably, lattice forces within the crystal could influence the conformation of the product formed by this two-phase method.

Because the second-shell contribution is less well resolved than that of the first shell (see Figure 2), analysis of the second-shell data is more tenuous. If for each imidazole ligand we assume that the Hg, the N, and the two flanking C's are coplanar and that the carbons are symmetrically placed about the Hg–N bond, then we would expect to see four carbons at 3.2–3.3 Å. Indeed, the best fit for this model (Table II) occurs for three to four carbons and is in good agreement with the crystallographic results for Hg plastocyanin.[23,37] Although there are no crystal structures

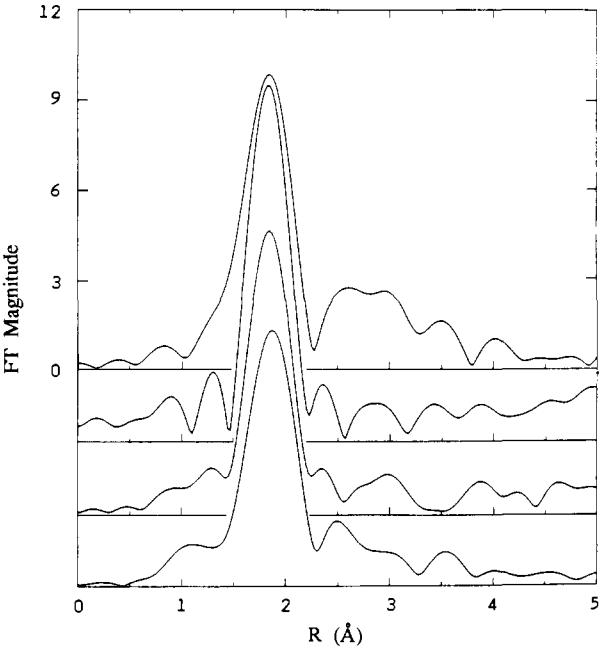

Figure 2. Fourier transforms of EXAFS data for Hg-substituted blue copper proteins. From top: azurin, plastocyanin, laccase, and stellacyanin. Transforms were calculated with k^3-weighted data over the range k = 3–12.8 Å^{-1}. Spectra have been displaced vertically for enhanced visibility.

available, the presence of two imidazole nitrogen ligands can safely be assumed to occur in stellacyanin[39] and laccase[40] in view of

(37) Strange et al. find multiple scattering to be important in describing metal–histidine EXAFS.[38] This does not bias our results since we have used empirical amplitude and phase parameters, which automatically include multiple scattering effects.

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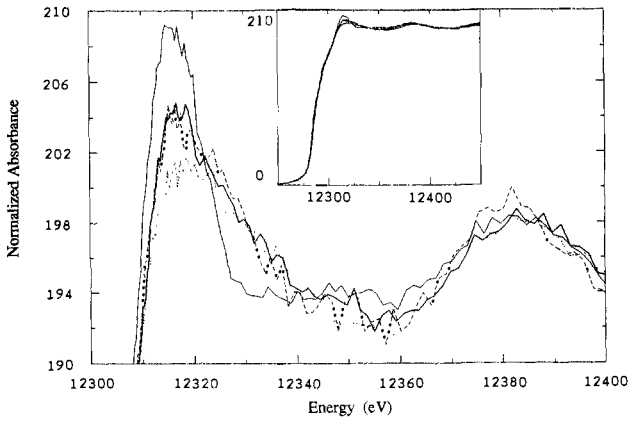

Figure 3. Details of Hg L_3 edge XANES spectra for Hg-substituted blue copper proteins. Light solid line (top), stellacyanin; dark solid line, plastocyanin; dashed line, azurin; dotted line, laccase. Spectra were normalized as described in the text. (**Inset**) Entire XANES region for the same spectra.

published spectroscopic results. For all four proteins, the second-shell data can also be fitted, with approximately the same quality of fit, by a model which assumes a single sulfur at about 3.0 Å in lieu of four carbons. The fact that the second shell can be fitted with sulfur only does not mean that the carbon atoms are absent; the carbon atoms must be present given the presence of nitrogen in the first shell. Rather, this result highlights the difficulties inherent in analyzing the (inevitably disordered) outer-shell EXAFS.[41]

In all cases, smaller residuals are calculated if instead of a single-atom model, we assume models which include either four carbons *and* one sulfur or four carbons *and* one or two oxygens. At the same time, the Debye–Waller factors increase in order to maintain an approximately constant EXAFS amplitude. Unfortunately, the improvements in the fits are not necessarily significant since we, in effect, double the number of adjustable parameters. The fit for Hg stellacyanin improves most markedly (Table II), and this finding, along with the XANES results discussed below, probably indicates that there is (are) additional scattering atom(s) in the second coordination shell of stellacyanin. Unfortunately, comparable fits are obtained by adding either sulfur or oxygen, so the curve fitting results do not enable us to identify the atom(s) involved. The problem can be understood by considering that the EXAFS waves from different scattering atoms are distinguished on the basis of their phase shifts, $\phi(k)$, and the shapes of their amplitude envelopes, $A(k)$. The difference in $\phi(k)$ for oxygen and sulfur accounts for the fact that R_{Hg-O} consistently refines to a distance 0.15–0.20 Å shorter than R_{Hg-S}. In principle, differences in $A(k)$ can distinguish sulfur from oxygen; however, this information is apparently lost in the correlation between Hg–X (X = O, S) and Hg–C EXAFS and in small adjustments of σ. Analogous difficulties were noted by Peisach et al.[42] in an EXAFS study of Cu stellacyanin. They found that the second-shell data could be fitted on the assumption of sulfur ligation but that the fit was not unique.

Differences in the XAS spectra due to variations in the axial ligation might be expected to occur in the XANES region, since the damping factors which prevent observation of weak interactions are less severe near the threshold energy. As can be seen in Figure 3, the XANES spectrum of Hg stellacyanin is unique in that it has a higher normalized peak amplitude at 12 317 eV and has a sharp decrease at 12 326 eV (i.e., a narrower principal maximum). The XANES spectrum for Hg laccase is similar to, although not identical with, that of Hg plastocyanin and Hg azurin. At the present time the theoretical understanding of the XANES region lags behind that of the EXAFS region, so we are unable to provide an unambiguous structural interpretation of these results; however, the variation in XANES structure clearly demonstrates that there is a difference in the coordination environment of Hg(II) in stellacyanin compared with those of the other proteins. Although XANES structure can be sensitive to geometry because of multiple scattering processes, these effects tend to be important in cases of collinear coordination (i.e., a Hg–X–Y angle of ca. 180°) or for very short ($R < 1.6$ Å) bonds,[43] Hence, it is unlikely that some type of deformation accounts for the spectrum.

The data are better explained in terms of a change in the axial ligation; however, the unique XANES spectrum of Hg stellacyanin is unlikely to relate to the presence or the absence of a distal peptide oxygen since the XANES spectra for Hg plastocyanin and Hg azurin are superimposable. One of the hypotheses in the literature is that stellacyanin may have a disulfide sulfur rather than a methionine sulfur in the axial position.[44–46] This substitution is attractive in that a disulfide sulfur could provide a ligand field similar to that of a methionine sulfur, thereby accounting for the similarities in the ligand-field spectra[47] and the ^{113}Cd NMR spectra.[44] If a cystine ligand is present, the XANES effect may actually relate to the disulfide sulfur which is β to Hg since the EXAFS results rule out an unusual, i.e., short, bond to the axial ligand. There is, of course, an alternative interpretation. Since we have to this point only detected a *change* in coordination, the XANES data could be signaling the absence of any type of axial sulfur ligand, a proposal that has also appeared in the literature.[48]

Conclusions

Analysis of the Hg EXAFS data from a series of Hg-substituted blue copper proteins reveals that the primary coordination shell of mercury contains a sulfur donor and two nitrogen donors. As was the case with Cu EXAFS,[42] it has not been possible to identify more weakly bonded ligands in the second coordination shell, even for stellacyanin. In the case of Hg plastocyanin, the EXAFS results are completely consistent with a previous crystallographic analysis which showed that the same basic coordination geometry is obtained whether copper or mercury is bound, although the metal to ligand bonds are elongated when mercury is present. The EXAFS data prove conclusively that mercury is bound selectively at the type 1 copper site in the mercury derivative of laccase. This work establishes the replacement of type 1 copper by mercury(II) to be a rational approach for the design and preparation of chemically useful mixed-metal derivatives of multi-copper proteins. The unique affinity of the type 1 site for mercury binding has previously been established through investigations of the thermal denaturation of azurin and azurin derivatives.[49] This affinity can be understood in terms of two important structural properties of type 1 copper: the presence of the mercaptide ligand and the low coordination number. Relativistic effects within the mercury atom dispose the ion to favor low coordination numbers.[50,51]

Finally, the XANES results confirm that the type 1 site in stellacyanin is structurally distinct from the other type 1 sites, and they indicate the potential that the analysis of the XANES

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region has for elucidating subtle changes in molecular structure. The similarities in the immediate ligation environment, as indicated by the first-shell data for stellacyanin and the other proteins, lead us to favor a model in which the variation in the XANES is attributed to changes in the (EXAFS nondetectable) axial ligand. With this in mind, it is interesting to note that the increase in absorbance at 12 317 eV (laccase < azurin, plastocyanin ≪ stellacyanin) parallels the decrease in reduction potential for the native (Cu containing) forms of these proteins, although a temperature-dependent conformational change in laccase could confuse the trend.[52] To the extent that the Cu and Hg sites are similar, our data may therefore be consistent with the proposal[5,53] that axial ligation plays an important role in tuning the reduction potential of the type 1 site. Model compound XANES studies are in progress in an attempt to understand and exploit the differences revealed in the spectra of the mercury-containing proteins.

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Registry No. Hg, 7439-97-6; L-histidine, 71-00-1; L-cysteine, 52-90-4.

Supplementary Material Available: Table of results pertaining to the wide-filter data and a listing of X-ray absorption vs energy for the spectra reported herein (25 pages). Ordering information is given on any current masthead page.

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A Specific Quadrilateral Synthesized from DNA Branched Junctions

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Abstract: Four different three-arm branched DNA junctions have been synthesized and covalently linked together in a prescribed arrangement to form a macrocycle of previously specified sequence. One end of each individual junction is closed by a hairpin loop. Each open arm of the four junctions terminates in a unique single-stranded cohesive ("sticky") end; there are four pairs of complementary sticky ends among the eight arms, two pairs with 3′ overhangs, and two pairs with 5′ overhangs. The associations between junctions are directed by the sequences of these cohesive ends. The junctions that associate in this fashion are enzymatically joined together by the use of bacteriophage T4 DNA ligase. The final product is a "quadrilateral" the sides of which are each 16 nucleotide pairs long, approximately 1.5 turns of DNA. The quadrilateral is designed to be composed of two hextuply linked circles of DNA.

There is presently considerable interest in the potential of biological molecules to provide new reagents and structural units for synthetic chemistry. One goal of protein engineering, for example, is to design and synthesize binding sites for selected ligands, introducing functional groups with appropriate geometry, which may lead to new catalysts. On the macromolecular scale, one can imagine affixing larger functional molecules, such as protein domains, in particular arrangements. A requirement for achieving this type of structural control is the ability to construct a scaffolding to position and orient the functional units. We show here that the stable double helix of DNA can be used in conjunction with stable DNA branched junctions to create designated closed structures that could function in this manner. Assembly of this model scaffold is directed by the specificity of Watson-Crick[1] base pairing.

DNA branched junctions are analogues of structures found as intermediates in recombination and replication (e.g., ref 2). They consist of three or more strands of DNA which associate to form three or more double-helical arms that flank a central branch site or "junction". We and others have characterized the structure and stability of three- and four-armed junctions.[3-10] In principle, these structures may be used as synthons for construction of N-connected polygonal and polyhedral stick figure devices and networks on the nanometer scale[11,12] rather than the Ångstrom scale more familiar to synthetic chemists (e.g., ref 13 and 14). This could be achieved by treating them as units to be linked together by the ligation techniques commonly employed in molecular biology.[15,16] From the standpoint of construction, line

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