Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355–360.
Berghäuser, J., & Schirmer, R. H. (1978) *Biochim. Biophys. Acta 537*, 454–463.
Egner, U., Tomasselli, A. G., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 649–658.
Ehresman, B., Imbault, P., & Weil, J. H. (1973) *Anal. Biochem. 54*, 454–463.
Feldhaus, P., Fröhlich, T., Goody, R. S., Isakov, M., & Schirmer, R. H. (1975) *Eur. J. Biochem. 57*, 197–204.
Fry, D. C., Kuby, S. A., & Mildvan, A. S. (1985) *Biochemistry 24*, 4680–4694.
Fry, D. C., Kuby, S. A., & Mildvan, A. S. (1987) *Biochemistry 26*, 1645–1655.
Kalbitzer, H. R., Marquetant, R., Rösch, P., & Schirmer, R. H. (1982).
Lienhard, G. E., & Secemski, I. I. (1973) *J. Biol. Chem. 248*, 1121–1123.
McDonald, G. G., Cohn, M., & Noda, L. (1975) *J. Biol. Chem. 250*, 6947–6954.
Noda, L. (1973) *Enzymes, 3rd ed. 8*, 279–305.
Rance, M., & Wright, P. E. (1986) *J. Magn. Reson. 66*, 372–378.
Rösch, P., & Gross, K. H. (1985) *J. Mol. Biol. 182*, 341–345.
Schulz, G. E., Elzinga, M., Marx, F., & Schirmer, R. H. (1974) *Nature (London) 250*, 120–123.
Schulz, G. E., Schiltz, E., Tomasselli, A. G., Frank, R., Brune, M., Wittinghofer, A., & Schirmer, R. H. (1986) *Eur. J. Biochem. 161*, 127–132.
Smith, G. M., & Mildvan, A. S. (1982) *Biochemistry 21*, 6119–6123.
Tomasselli, A. G., & Noda, L. H. (1980) *Eur. J. Biochem. 103*, 481–491.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids* Wiley, New York.

The Copper Sites of Dopamine β-Hydroxylase: An X-ray Absorption Spectroscopic Study†

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ABSTRACT: X-ray absorption edge and extended X-ray absorption fine structure (EXAFS) spectra are reported for the Cu(I) and Cu(II) forms of bovine dopamine β-hydroxylase (DBH; EC 1.14.17.1) and for the Cu(I) form of DBH bound either to tyramine substrate or to a multisubstrate inhibitor [Kruse, L. I., DeWolf, W. E., Jr., Chambers, P. A., & Goodhart, P. J. (1986) *Biochemistry 25*, 7271–7278]. A significant change in the structure of the copper sites occurs upon ascorbate-mediated reduction of Cu(II) DBH to the Cu(I) form. While the average Cu(II) site most likely consists of a square-planar array of four (N,O)-containing ligands at 1.98 Å, the average Cu(I) site shows a reduction in (N,O) coordination number (from ~4 to ~2) and the addition of a S-containing ligand at 2.30 Å. No change in the average Cu(I) ligand environment accompanies binding of tyramine substrate, whereas binding of a multisubstrate inhibitor, 1-(3,5-difluoro-4-hydroxybenzyl)-1*H*-imidazole-2(3*H*)-thione, causes an increase in the Cu–S coordination, consistent with inhibitor binding to the Cu(I) site through the S atom. Although excellent signal-to-noise ratio in the EXAFS spectra of ascorbate-reduced DBH facilitated analysis of outer-shell scattering for a Cu···Cu interaction, the presence of a binuclear site could not be proven or disproven due to interference from Cu···C scattering involving the carbons of imidazole ligands.

Dopamine β-hydroxylase (DBH;[1] EC 1.14.17.1) is a copper-containing monooxygenase that catalyzes the benzylic hydroxylation of dopamine to norepinephrine (Skotland & Ljones, 1979; Rosenberg & Lovenberg, 1980; Villafranca, 1981; Ljones & Skotland, 1984). In spite of the physiological importance of this biotransformation (Kruse et al., 1986a) and the interesting dependence of the monooxygenase activity upon prosthetic copper ions, surprisingly little is known about the ligand environment of the active site copper atoms in DBH (Kruse et al., 1986b). Indeed, even the copper:protein stoichiometry has been an issue for debate although recent studies have convincingly demonstrated that a 2:1 copper:subunit stoichiometry leads to maximal catalysis for phenethylamine substrate (Klinman et al., 1984) or alternative substrates (Ash et al., 1984). Catalysis by DBH follows a priming of the enzyme by reduction of the Cu(II) form to the Cu(I) form by a one-electron donor (Rosenberg & Lovenberg, 1980;

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[1] Abbreviations: DBH, dopamine β-hydroxylase; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structure; FT, Fourier transform; HPLC, high-performance liquid chromatography; XAS, X-ray absorption spectroscopy.

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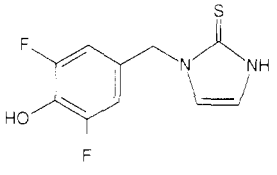
Ljones & Skotland, 1984). The net two-electron reaction stoichiometry, a 2:1 copper:subunit ratio, and a functional relationship to binuclear copper proteins such as tyrosinase (Wilcox et al., 1985) or hemocyanin (Solomon, 1981; Preaux & Gielens, 1984) would seem to argue in support of a binuclear copper site. In contrast, EPR studies of the resting Cu(II) form of DBH with highly variable copper content show no evidence for magnetic interaction between the copper atoms (Blumberg et al., 1965; Friedman & Kaufman, 1966; Walker et al., 1977; Skotland et al., 1980; Ash et al., 1984). A previous EXAFS study on the Cu(II) form of DBH (Hasnain et al., 1984) showed no evidence for a Cu···Cu interaction, although the data extended only to $k = 9$ Å^{-1} and the DBH sample contained substoichiometric copper [3.95 mol Cu(II)/mol tetramer].

Here we report the results of XAS experiments on highly concentrated (~8 mM in Cu) samples of the Cu(II) and Cu(I) oxidation states of bovine DBH with 2:1 copper:subunit stoichiometry. We also report the results of XAS experiments on the Cu(I) form of DBH when bound to tyramine substrate and the previously reported (Kruse et al., 1986b) multisubstrate inhibitor 1-(3,5-difluoro-4-hydroxybenzyl)-1*H*-imidazole-2(3*H*)-thione (I).



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Experimental Procedures

Materials

Bovine (cow, steer) adrenal glands were obtained at a slaughterhouse and placed into an isotonic (0.9%) NaCl water/ice slush within 30 min of death. "Buffer" refers to 5 mM potassium phosphate, pH 6.5. Other materials were obtained from the following suppliers: Boehringer-Mannheim Biochemicals, crystalline catalase (sp act. 65 000 units/mg as claimed by supplier); BRL, $(NH_4)_2SO_4$; Pharmacia, concanavalin A–Sepharose; Calbiochem-Behring, methyl α-D-mannoside; Pierce, Whatman DE-52; Bio-Rad, all chemicals used for gel electrophoresis; Sigma, tyramine hydrochloride, DL-octopamine, disodium fumarate; Aldrich, L-ascorbic acid. 1-(3,5-Difluoro-4-hydroxybenzyl)-1*H*-imidazole-2(3*H*)-thione was prepared as previously reported (Kruse et al., 1986b). All other chemicals were of the highest grade available.

Enzyme Preparation (Table I)

With the exception of the HPLC step, all operations were carried out at 4 °C. The enzyme isolation from 2–3 lb of adrenal glands is described although 15–20 lb was used in a typical large-scale isolation.

Dissection and Homogenization of Medullae. The dissection of medullae from 2–3 lb of adrenal glands was completed within 4 h of death. The dissected tissue was placed into buffer (4 mL/g of tissue) that contained 100 μg/mL crystalline catalase (Rush et al., 1974; Aunis et al., 1975). The medulla/buffer mixture was homogenized for 5 min at low speed in a Waring blender and centrifuged (100000*g*, 30 min) to give a crude extract. The resulting pellet was discarded.

Adsorption to Concanavalin A–Sepharose. The crude extract (typically 400–600 mL) was stirred gently for 2 h with 30 mL (packed volume) of concanavalin A–Sepharose. The Sepharose was then filtered through a coarse sintered funnel and poured into a 1.6 × 20 cm column. The column was washed (2 mL/min) with 300 mL of 0.2 M NaCl in buffer, and the enzyme was eluted (0.2 mL/min) with 10% methyl α-D-mannoside and 0.2 M NaCl in buffer. Fractions of 5 mL were collected, and those with a significant absorbance at 280 nm were pooled (typically 100 mL) and brought to 70% saturation with $(NH_4)_2SO_4$. The suspension was centrifuged (12000*g*, 20 min), and the protein pellet was dissolved in 5–10 mL of buffer.

DEAE Ion-Exchange Chromatography. The concentrated protein solution was desalted by dialysis against two 1-L changes (3 h each) of buffer and then applied to a 1.6 × 10 cm column of DE-52 that had been previously equilibrated with buffer. The column was washed with buffer until the eluent had a low absorbance at 280 nm, and the protein was eluted with a linear 0–0.7 M NaCl gradient in buffer. The fractions that contained significant DBH activity were pooled and brought to 70% saturation with $(NH_4)_2SO_4$. The suspension was centrifuged (12000*g*, 20 min), and the protein pellet was dissolved in 5–10 mL of buffer.

Preparative Size-Exclusion HPLC. A Bio-Rad TSK-250 22.5 × 600 mm preparative size-exclusion HPLC column was equilibrated with 0.2 M KCl in buffer. Injections (5 mL) of the concentrated protein solution were made, the column was eluted at 8 mL/min with 0.2 M KCl in buffer, and the fractions that contained significant DBH activity were pooled and brought to 70% saturation with $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ suspension was stored at 4 °C.

Methods

Assay for Enzymatic Activity. Enzymatic activity was determined by the conversion of tyramine to octopamine in an assay similar to the procedure of Nagatsu and Udenfriend (1972). Enzymatic activity of homogeneous DBH was also determined by oxygen consumption according to the procedure previously described (Kruse et al., 1986b). Conditions for the octopamine assay were as follows: 0.2 M sodium acetate, pH 5.0, 10 μM $CuCl_2$, 1 mg/mL crystalline catalase, 10 mM ascorbate, 10 mM sodium fumarate, 1 mM pargyline, 30 mM *N*-ethylmaleimide, and 10 mM tyramine. Incubations were carried out at 37 °C in a reciprocating shaker–bath. Conditions for the oxygen electrode assay were as follows: 0.2 M sodium acetate, pH 5.0, 1 mg/mL crystalline catalase, 10 mM ascorbate, 10 mM sodium fumarate, 10 mM tyramine, and 0.24 mM oxygen.

Assay for Protein Concentration. Protein concentration was determined spectrophotometrically by using the previously reported $A_{280}(1\%) = 12.4$ (Skotland & Ljones, 1977).

Gel Electrophoresis. Nondenaturing gels (1.5-mm slab) were run at a 5% polyacrylamide concentration with no stacking gel. SDS–polyacrylamide gels (Laemmli, 1970) (1.5-mm slab) were run at a 10% polyacrylamide concentration with a 4% stacking gel. Protein was visualized with silver stain.

Determination of Copper. Copper was determined on enzyme samples diluted into buffer, by using an ICP 2500 (Plasma-Therm, Inc.) connected to an Instruments SA JY38 sequential spectroanalyzer. A 1000 ppm solution of copper sulfate was used as reference standard.

Determination of Chloride. The amount of chloride in the XAS samples was determined by ion chromatography using a Dionex QIC ion chromatograph equipped with a Dionex HPIC A64 guard column, a Dionex HPIC A54 separator column, an anion micromembrane suppressor, and a conductivity detector. The eluent was 2 mM $NaHCO_3$.

Preparation of Samples for XAS. Purified DBH (Table I) having a specific activity of 28–30 units/mg (typically >100

Table I: Purification of Dopamine β-Hydroxylase from Whole Medulla

purification step	protein[a,b]	act.[b,c]	sp act.[b] (U)	x-fold purification	yield (%)
crude lysate	62	9.4 ± 1.3	0.15 ± 0.02		100
concanavalin A	1.3 ± 0.2	5.5 ± 0.7	4.6 ± 0.5	31	59
DEAE	0.42 ± 0.11	3.4 ± 0.4	7.8 ± 0.6	52	36
HPLC	0.11 ± 0.04	2.5 ± 0.5	23 ± 4	150	27

[a] Expressed as mg of protein/g wet weight of starting medulla. [b] Standard errors are indicated for multiple isolations. [c] Expressed as units of activity/g wet weight of medulla.

mg) as the $(NH_4)_2SO_4$ suspension was centrifuged at 10000*g* for 10 min. The supernatant was removed and the pellet was dissolved in 5–10 mL of buffer. The concentration of DBH subunit was determined from the absorbance of the solution at 280 nm and the estimated molecular weight of 70 000. The enzyme was then reconstituted, through the addition of 5 mM $CuCl_2$, with 2 mol equiv of Cu(II). Dialysis followed vs 3 × 2 L of 50 mM sodium phosphate, pH 6.6, containing 5 μM $CuCl_2$ for a total of 9 h. The DBH solution was concentrated to less than 0.5 mL by using a collodion bag apparatus (Schleicher & Schuell) and a membrane having a molecular weight cutoff of 75 000. At this stage, protein and copper determinations indicated a Cu(II):subunit ratio of 2.04. The concentrated enzyme was then placed in Lucite sample cuvettes (Scott et al., 1986). Other additions as necessary were made directly into the cuvettes. Reduction of the appropriate sample was achieved by the addition of 1.0 M sodium ascorbate to give a final concentration of 50 mM. The tyramine-treated reduced DBH sample was prepared by adding a solution containing 0.5 M sodium ascorbate and 0.1 M tyramine hydrochloride to give final concentrations of 50 and 10 mM, respectively. To prepare the inhibitor-treated reduced DBH sample, a solution containing 0.33 M sodium ascorbate and 67 mM 1-(3,5-difluoro-4-hydroxybenzyl)-1*H*-imidazole-2-(3*H*)-thione (I, sodium salt) was added to give final concentrations of 50 and 10 mM, respectively. To ensure complete reduction and mixing of the samples, the solutions were added evenly along the long edge of the cuvette, followed by gentle mixing with the needle of the Hamilton syringe. Upon reduction, the samples changed from a blue-green to a yellow-green color. All samples were frozen and stored in liquid nitrogen immediately following preparation. After EXAFS and EPR experiments, assay of the recovered enzyme showed no detectable loss of specific activity.

X-ray Absorption Spectroscopy. All XAS data were collected at the Stanford Synchrotron Radiation Laboratory on the wiggler beam line VII-3 with the SPEAR ring operating under dedicated conditions (3.0 GeV, ~60 mA). The X-ray beam was monochromated by using Si[220] crystals, and energy calibration was performed according to the internal calibration technique (Scott, 1985) with 5 μm thick copper foil as a reference. The data were collected by fluorescence excitation using an argon-filled ionization chamber fluorescence detector (EXAFS Co., Seattle) on samples maintained at 4 or 10 K as frozen solutions in a custom-made Oxford Instruments cryostat (now Model CF1208). Each EXAFS spectrum consists of 10 signal-averaged 23-min scans covering the 8650–9700-eV range, whereas each high-resolution edge spectrum required signal averaging of only 2–3 15-min scans.

Data reduction and analysis were accomplished by our standard methods (Scott et al., 1986). Averaged spectra (F/I_0) were formed, and the background was subtracted by fitting a second-order polynomial to the data in the region 9050–9650 eV, adjusted by a constant to match the data just before the edge (8950 eV). The EXAFS data were extracted from the resultant data by fitting a cubic spline over the range 9028–9650 eV (spline points at 9180 and 9400 eV), subtracting, and normalizing the resultant data to the atomic falloff modeled by the Victoreen formula, normalized to match the spline at $k = 0$ Å^{-1} ($E_0 = 9000$ eV).

Curve fitting of first-shell atoms employed backscattering (phase and amplitude) functions empirically derived from model compounds by complex Fourier back-transformation (Scott, 1985). For Cu(II)–N and Cu(II)–S, these functions were extracted from a series of Cu(II) compounds as previously described (Scott et al., 1986). For Cu(I)–N, the compound $[Cu^I(BBDHp)](PF_6)_x(BF_4)_{1-x}$ [BBDHp = 1,7-bis(2-benzimidazolyl)-2,6-dithiaheptane] (Schilstra et al., 1982) was used, and for Cu(I)-S, $[Cu(etu)_3](SO_4)_{1/2}$ (etu = ethylenethiourea) (Weininger et al., 1972) was used. For outer-shell Cu···C interactions, a model compound was required that exhibits well-defined sets of outer-shell carbons, preferably from imidazole-like ligands (since imidazoles are probable ligands to copper in DBH; vide infra). $[Cu(Im)_4]^{2+}$ (Im = imidazole) and other square-planar, imidazole-containing complexes are not satisfactory, owing to the presence of axial ligands (H_2O or counterions) at longer distances that interfere with the Cu···C shell. The nearly tetrahedral complex $[Cu\{(Im)_2bph\}_2](ClO_4)_2$ [$(Im)_2bph$ = 2,2′-diimidazolylbiphenyl] (Knapp et al., 1987) was chosen, and a sample was kindly provided by H. J. Schugar. The slight tilting of imidazole rings in this Cu(II) compound results in the presence of three sets of Cu···C interactions: 4C's at 2.86 Å; 12 C's at 3.15 Å; 16 C's at 4.13 Å. The last set was treated separately, and a FT peak at $R' = 3.6$ Å was used to extract (by complex Fourier back-transformation) scattering functions for Cu···C interactions with R(Cu···C) ≈ 3.5–4.5 Å. The other two sets (at 2.86 and 3.15 Å) combine to give rise to a single FT peak at $R' = 2.6$ Å. The lack of resolution required a different approach for extraction of scattering parameters for shorter distance (R ≈ 2.5–3.5 Å) Cu···C interactions. We used a technique similar to the FABM (fine adjustment based on models) method (Teo et al., 1983), in which curve fitting of filtered data was performed by using two Cu···C shells simulated with theoretical scattering functions (Teo & Lee, 1979). The best fits were obtained with $B_s = 0.3$ [see eq 1 of Scott et al. (1986)] and $\Delta E_0 = +5$ eV, and these values were then used with theoretical scattering functions to fit the DBH data.

In order to explore the possible existence of a binuclear copper site in ascorbate-reduced DBH, Cu···Cu scattering functions were extracted from $[Cu(salen)]_2$ (salen = *N*,*N*′-disalicylideneethylenediamine), which displays a stacked-dimer structure with a Cu···Cu distance of 3.18 Å (Hall & Waters, 1960). A sample of $[Cu(salen)]_2$ was kindly provided by I. Morgenstern-Badarau. The FT of the Cu EXAFS of this compound exhibits a peak at $R' = 2.7$ Å that contains contributions from Cu···Cu as well as six Cu···C at 2.96 Å and three Cu···C at 3.29 Å. Using the Cu···C scattering functions described above to simulate the two Cu···C shells and the theoretical scattering functions for Cu···Cu led to $B_s = 1.0$ and $\Delta E_0 = +25$ eV as the best-fit parameters for Cu···Cu. Using these parameters and the theoretical Cu···Cu scattering functions on a number of structurally characterized copper dimers[2] yielded Cu···Cu distances that were consistently ~0.08

Table II: Curve-Fitting Results for the First Coordination Sphere of Dopamine β-Hydroxylase Derivatives[a]

sample	fit	Cu–(N,O) N_s	Cu–(N,O) R_{as} (Å)	Cu–(N,O) $\Delta\sigma_{as}^2$ (Å^2)	Cu–(S,Cl) N_s	Cu–(S,Cl) R_{as} (Å)	Cu–(S,Cl) $\Delta\sigma_{as}^2$ (Å^2)	f'[b]
oxidized	1	(4)[c]	1.98	+0.0019				0.019
reduced, sample A	2	(2)	1.93	+0.0032	(1)	2.30	−0.0008	0.026
	3	(3)	1.94	+0.0066	(1)	2.32	−0.0009	0.040
reduced, sample B	4	(2)	1.92	+0.0040	(1)	2.30	+0.0002	0.039
	5	(3)	1.92	+0.0074	(1)	2.32	−0.0001	0.050
reduced, +tyramine	6	(2)	1.93	+0.0042	(1)	2.30	+0.0002	0.026
	7	(3)	1.94	+0.0077	(1)	2.32	+0.0002	0.044
reduced, +inhibitor	8	(1)	1.96	+0.0012	(2)	2.30	+0.0025	0.027
	9	(2)	1.97	+0.0068	(2)	2.31	+0.0030	0.044
	10	(2)	2.00	+0.0046	(1)	2.32	−0.0022	0.022
	11	(1.5)	1.98	+0.0032	(1.5)	2.31	+0.0002	0.020

[a] N_s is the number of scatterers per copper; R_{as} is the copper–scatterer distance; $\Delta\sigma_{as}^2$ is a relative mean square deviation in R_{as}, $\Delta\sigma_{as}^2 = \sigma_{as}^2$(sample) − σ_{as}^2(model). The model compounds are described under Experimental Procedures. [b] f' is a goodness-of-fit statistic normalized to the overall magnitude of the $k^3\chi(k)$ data (Scott et al., 1986): $f' = \{\sum[k^3[\chi_{obsd}(i) - \chi_{calcd}(i)]]^2/N\}^{1/2}/[(k^3\chi)_{max} - (k^3\chi)_{min}]$. [c] Numbers in parentheses were not varied during optimization.

Å shorter than the crystallographic distances.

For Cu X-ray absorption edge comparisons (see Figure 1), some related model compounds and copper enzymes were examined. The data for $[Cu(Im)_4]^{2+}$ were available from a previous study (Scott & Dooley, 1985), as were the data for the Cu(II) form of bovine plasma amine oxidase. The dithionite-reduced [Cu(I)] sample of bovine plasma amine oxidase was prepared by D. M. Dooley. $[Cu^I(BBDHp)](PF_6)_x(BF_4)_{1-x}$ was a gift from J. Reedijk (Schilstra et al., 1982). The data for $[Cu^I(L_1\text{-Pr})](BF_4)$ [L_1-Pr = 2,2′-bis-[2-(*N*-propylbenzimidazolyl)]diethyl sulfide] (Dagdigian et al., 1982) were kindly provided by L.-S. Kau (Kau et al., 1987).

Electron Paramagnetic Resonance (EPR) Spectroscopy. EPR spectra were recorded on a Bruker ER 200D instrument equipped with a TE102 cavity and an Oxford ER-9 cryostat which is available within the Center for Metalloenzyme Studies. The spectra were recorded on samples in the Lucite XAS cuvettes which are designed for in situ EPR measurements (Scott et al., 1986). The microwave frequency and power were 9.42 GHz and 4 mW, respectively. The modulation frequency and amplitude were 100 kHz and 12.5 G, respectively. The sample temperature was 90 K. The relative concentrations of Cu(II) in the ascorbate-reduced DBH samples were measured by comparison of the lowest field $A_\parallel$ component with the same component of a fully oxidized DBH sample of identical concentration. All ascorbate-reduced samples were found to be ≥94% reduced by this measurement.

Results

Copper Edges. The copper K X-ray absorption edge spectra for oxidized and ascorbate-reduced forms of DBH are compared in Figure 1 with edge spectra for similar forms (oxidized and dithionite reduced) of bovine plasma amine oxidase and for the "model" compounds $[Cu(Im)_4]^{2+}$, $[Cu(L_1\text{-Pr})]^+$, and $[Cu(BBDHp)]^+$. Comparison of edges for DBH enzyme forms (Figure 1b) with the edges for amine oxidase (Figure 1a) confirms the presence of only Cu(II) in the oxidized form and virtually complete reduction to Cu(I) in the ascorbate-reduced form. The edge for the Cu(II) form of DBH is very similar to the edge for oxidized amine oxidase and exhibits features in common with the edge of $[Cu(Im)_4]^{2+}$ (Figure 1c), fully

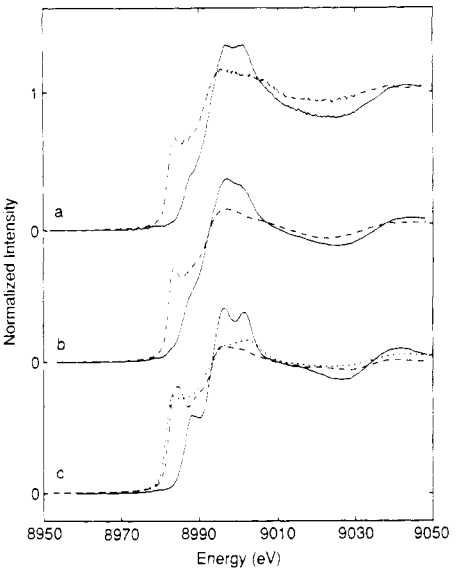

FIGURE 1: Comparison of Cu K absorption edges for (a) oxidized (—) and dithionite-reduced (- - -) bovine plasma amine oxidase; (b) oxidized (—) and ascorbate-reduced (- - -) bovine dopamine β-hydroxylase; and (c) $[Cu^{II}(Im)_4]^{2+}$ (—), $[Cu^I(BBDHp)]^+$ (- - -), and $[Cu^I(L_1\text{-Pr})]^+$ (···).

consistent with predominantly imidazole ligation (Scott & Dooley, 1985). Since the edges in Figure 1b result from an average copper environment, no information is available regarding the possible differences between the structures of the two copper sites.

Copper EXAFS. Figure 2 and Table II summarize our analysis for the Cu EXAFS of oxidized and ascorbate-reduced forms of DBH. The Cu EXAFS and Fourier transform (FT) of oxidized DBH (Figure 2a,b) have appearances reminiscent of data obtained from $[Cu(Im)_4]^{2+}$ (Co et al., 1981; Scott & Dooley, 1985). FT peaks between $R' \approx 2.0$–3.8 Å (Figure 2b) are most likely due to scattering by carbons and nitrogens in the outer shells of imidazole rings, indicating coordination by histidine (vide infra). Curve-fitting analysis of the extracted first-shell FT peak (Figure 2c) yields the result shown in Table II: 4 ± 1 (N,O)-containing ligands with Cu–(N,O) distances of 1.98 ± 0.02 Å.

Comparison of Figure 2a,d indicates that a very significant copper site structural change accompanies ascorbate reduction. We examined two separate samples (A and B) of ascorbate-reduced DBH to confirm these changes (Figure 3 and Table II); the data from sample A are given in Figure 2. The Cu EXAFS FT of ascorbate-reduced DBH exhibits two main (first-shell) peaks at $R' \approx 1.5$ and 1.9 Å (peaks 1 and 2 in

[2] The following copper binuclear complexes were examined (Scott and Eidsness, unpublished results): $[Cu_2(L')(OH)]^{2+}$, complex IIIB of Karlin et al. (1984); $Cu_2[(fsa)_2en]$, where $[(fsa)_2en]^{4-}$ is the Schiff base *N*,*N*′-(2-hydroxy-3-carboxybenzylidene)-1,2-diaminoethane (Kahn et al., 1982); $[Cu_2(L\text{-Et})(OAc)]^{2+}$, where HL-Et is *N*,*N*,*N*′,*N*′-tetrakis[2-(1-ethylbenzimidazolyl)]-2-hydroxy-1,3-diaminopropane (McKee et al., 1984).

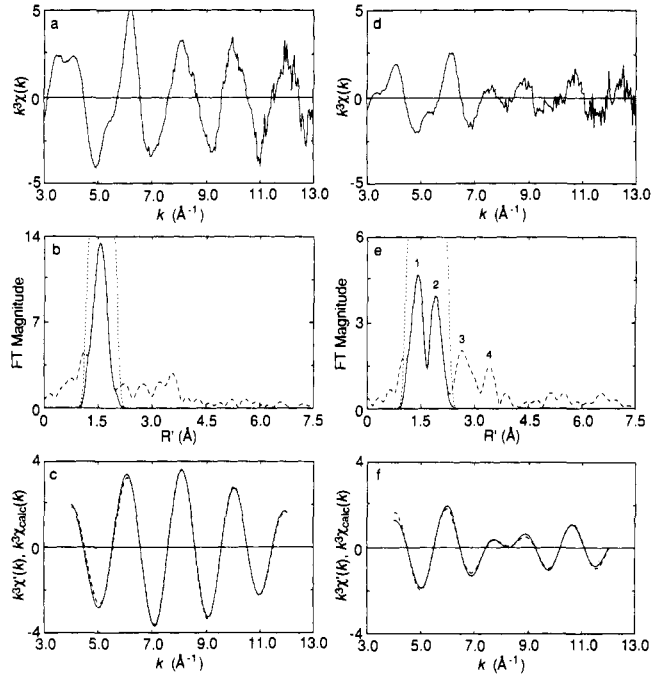

FIGURE 2: Comparison of Cu EXAFS analyses for the oxidized [Cu(II), left] and ascorbate-reduced [Cu(I), sample A, right] forms of dopamine β-hydroxylase. Fourier transforms (k = 3.0–13.0 Å^{-1}, k^3 weighting) of the raw Cu EXAFS data [in (a) and (d)] are shown in (b) and (e), respectively. The filter windows (···) in (b) and (e) were used to extract the first-shell contributions that are shown as back-transformed $\chi'(k)$ data (—) in (c) and (f), respectively. The best curve-fitting results are shown as dashed lines in (c) and (f), corresponding to fits 1 and 2 in Table II, respectively.

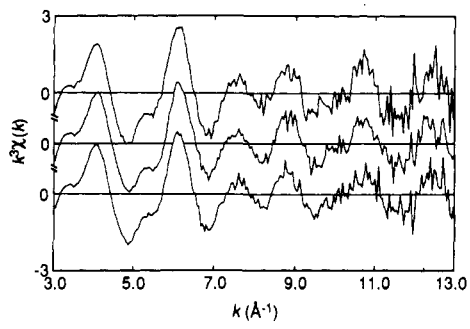

FIGURE 3: Comparison of raw Cu EXAFS data for the Cu(I)- (sample A, top; sample B, middle) and the Cu(I)–tyramine-bound (bottom) forms of dopamine β-hydroxylase. All three spectra are identical to within the noise of the data.

Figure 2e). Extraction and curve-fitting analysis of peaks 1 and 2 (Figure 2f and Table II) indicate that they arise from Cu–(N,O) and Cu–S interactions, respectively. As summarized in Table II, the best fits are obtained by assuming two or three (N,O)-containing ligands with Cu–(N,O) distances of 1.93 ± 0.02 Å and one S-containing ligand with a Cu–S distance of 2.30 ± 0.02 Å.

Figure 4 and Table II provide an analysis of two additional ascorbate-reduced samples of DBH, one with tyramine substrate bound and the other with the multisubstrate inhibitor I bound. Figure 3 shows that the raw Cu EXAFS data look identical for ascorbate-reduced DBH with and without added tyramine. The data analysis in Table II also demonstrates that binding of tyramine by the Cu(I) form of DBH causes no significant change in the copper atom environment. Binding of the multisubstrate inhibitor produces a significant change in the Cu(I) environment, as evidenced by the relative magnitudes of the FT peaks at R' = 1.4 and 1.8 Å (Figure 4b vs Figure 4e). The FT suggests and the curve-fitting results

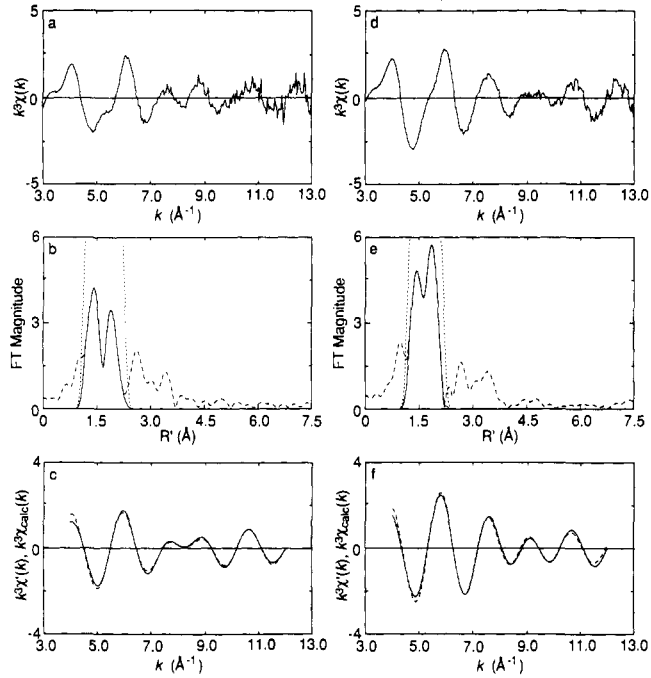

FIGURE 4: Comparison of Cu EXAFS analysis for the Cu(I)–tyramine)-bound (left) and Cu(I)–inhibitor-bound (right) forms of dopamine β-hydroxylase. The analyses were carried out as described for Figure 2. The best curve-fitting results (---) in (c) and (f) correspond to fits 6 and 8 in Table II, respectively.

(Table II) confirm an increase in sulfur ligation. Fit 11 (Table II), in which the two Cu(I) sites are treated independently, is best in terms of both f' and Cu–(N,O) and Cu–(S,Cl) $\Delta\sigma_{as}^2$ values (compared to other best fits in Table II).

The possibility that a chloride bound to copper could be responsible for the scattering component that we have assigned as Cu–S in the EXAFS of the reduced and tyramine-bound reduced DBH samples prompted us to perform chloride determinations on the XAS samples. The amount of chloride for various samples ranged from 0.23 to 0.64 Cl^-/Cu with an average and standard deviation of 0.39 ± 0.22 Cl^-/Cu. Since our curve-fitting results strongly suggest a (S,Cl) coordination number of 1 (the $\Delta\sigma_{as}^2$ values in Table II are very close to 0), we prefer to assign this scattering component to a Cu–S interaction.

To investigate the possible existence of a binuclear copper site in ascorbate-reduced DBH, the Cu EXAFS data for both samples A and B (see Table II) were averaged and the FT peaks analogous to peaks 3 and 4 in Figure 2e were separately analyzed for the presence of Cu···Cu scattering. Since imidazoles are expected to be coordinated to Cu(I), the Cu···C scattering from the carbons in the imidazole rings will contribute to *both* of these FT peaks, making the search for Cu···Cu scattering difficult. With Cu···C and Cu···Cu scattering functions generated as discussed under Experimental Procedures, the criterion used for establishing the presence of Cu···Cu was a requirement for a Cu···Cu contribution in fits of the Fourier-filtered peaks 3 or 4 which also involved Cu···C contributions. Table III summarizes the results for fits in which a total of four Cu···C interactions were assumed in each peak (i.e., two imidazoles per copper). FT peak 3 can be attributed equally well to a statically disordered set of Cu···C interactions (with R = 2.94, 3.34 Å) or to a Cu···Cu interaction (with R = 3.18 Å). Addition of a Cu···C interaction to this Cu···Cu interaction does not significantly improve the fit. FT peak 4 can be fit with two shells: R(Cu···C) = 3.96 Å, or R(Cu···Cu) = 3.78 Å. A fit with two Cu···C in-

Table III: Curve-Fitting Results for Outer Coordination Shells of Ascorbate-Reduced Dopamine β-Hydroxylase Derivatives[a]

		shell 1				shell 2				
FT peak[b]	fit	s	N_s	R_{as} (Å)	$\Delta\sigma_{as}^2$ (Å^2)	s	N_s	R_{as} (Å)	$\Delta\sigma_{as}^2$ (Å^2)	f'[c]
3	1	C	(2)[d]	2.94	−0.0014	C	(2)	3.34	+0.0011	0.089
	2	Cu	(1)	3.18	+0.0103					0.099
4	3	C	(1)	3.57	−0.0014	C	(3)	3.97	−0.0007	0.120
	4	Cu	(1)	3.78	+0.0229	C	(4)	3.96	+0.0016	0.090

[a] s is the scattering atom; N_s is the number of scatterers per copper; R_{as} is the copper–scatterer distance; $\Delta\sigma_{as}^2$ is a relative mean square deviation in R_{as}, $\Delta\sigma_{as}^2 = \sigma_{as}^2$(sample) − σ_{as}^2(model). For fit 1, σ_{as}^2(model) was taken as the vibrational component of the σ^2 from the appropriate Cu···C shell of $[Cu\{(Im)_2bph\}_2]^{2+}$, the static component being calculated (Scott, 1985) from the structure. For the shorter Cu···C shell, σ_{vib} = 0.052 Å; for the longer, σ_{vib} = 0.044 Å. For fits 2 and 4, the Cu···Cu model is $[Cu(salen)]_2$ [σ(model) = 0.062 Å]. For fits 3 and 4, σ_{as}^2(model) was taken as the vibrational component (σ_{vib} = 0.040 Å) from the ~4-Å Cu···C shell of $[Cu\{(Im)_2bph\}_2]^{2+}$. [b] The peak from the FT of the averaged Cu EXAFS from samples A and B of ascorbate-reduced DBH, labeled as shown in Figure 2e. [c] f' is defined in Table II. [d] Numbers in parentheses were not varied during optimization.

teractions does almost as well, although an uneven distribution of carbons is required (1 C at 3.57 Å; 3 C's at 3.97 Å). The large $\Delta\sigma^2$ for the Cu···Cu interaction may be indicative of larger inelastic scattering losses at these longer distances.

Discussion

The average copper site structure in the Cu(II) form of DBH is rather similar to the structure of the nonblue copper site in bovine (or porcine) plasma amine oxidase, which contains predominantly N-bonded heterocyclic ligands (either histidine imidazoles or the pyrroloquinoline quinone cofactor) and one water as equatorial ligands (Scott & Dooley, 1985; Baker et al., 1986). The shoulder observed at ~8988 eV in the Cu(II) K X-ray absorption edges for both these enzymes and for $[Cu(Im)_4]^{2+}$ (Figure 1) is probably assignable to a 1s → $4p_z$ transition (Smith et al., 1985). This peak is expected to be well separated from the main edge for square-planar geometries, and it is possible that the lack of separation exhibited in the edges of Figure 1 is due to the presence of weakly bound axial ligands in a Jahn–Teller distorted d^9 Cu(II) site. An axial water ligand has been proposed for both amine oxidase (Baker et al., 1986) and DBH (Obata et al., 1987).

Ascorbate reduction of DBH to the Cu(I) form results in a major structural change in the average copper site. The average Cu(I) site still contains some imidazole ligands but also one sulfur-containing ligand at a Cu(I)–S distance similar to that found in reduced cytochrome *c* oxidase (Scott, 1982). The intense preedge feature at ~8982 eV in the edge of ascorbate-reduced DBH (Figure 1b) is also observed in the edges of the two Cu(I) "models" in Figure 1c. $[Cu(L_1\text{-}Pr)]^+$ has a planar, T-shaped N_2S coordination (Dagdigian et al., 1982), whereas $[Cu(BBDHp)]^+$ has a linear N_2 coordination (Schilstra et al., 1982). Four- or five-coordinate Cu(I) compounds do not exhibit this intense preedge feature at this energy (Kau et al., 1987), suggesting a low- (probably three-) coordinate structure for the Cu(I) site in ascorbate-reduced DBH (*and* in dithionite-reduced bovine plasma amine oxidase). The shape of these Cu(I) edges (Figure 1c) is relatively insensitive to the proportions of N and S ligation. The curve-fitting results suggest either three- or four- coordinate Cu(I), but the Cu(I)–(N,O) distances are similar to Cu–N(imidazole) distances observed in structurally characterized low-coordinate Cu(I) complexes (Karlin et al., 1984; Dagdigian et al., 1982; Schilstra et al., 1982). Thus, the edge and EXAFS data in combination suggest $(N,O)_2S$ coordination for the average Cu(I) site in ascorbate-reduced DBH.

We are unable to determine with the reported XAS data whether a binuclear copper center exists in ascorbate-reduced DBH. The presence of Cu···C interactions from Cu(I)–histidine coordination masks the presence of a possible Cu···Cu FT peak. Potential tilting of the imidazole rings makes statically disordered Cu···C distances a viable possibility, further complicating our determination of Cu···Cu by curve-fitting analysis. The results in Table III reveal the following three possibilities: (a) a binuclear site with a Cu(I)–Cu(I) distance of ~3.2–3.3 Å (including the known distance error in these Cu···Cu scattering functions; see Experimental Procedures); (b) a binuclear site with a Cu(I)–Cu(I) distance of ~3.8–3.9 Å; or (c) two isolated Cu(I) sites [i.e., the Cu(I)–Cu(I) distance is greater than 4 Å]. Further XAS studies with structurally characterized copper binuclear compounds may eventually allow us to choose from these three possibilities.

The binding of tyramine substrate to the ascorbate-reduced Cu(I) form of DBH has no effect upon the copper ligand environment despite a (presumed) close spatial relationship to the catalytically competent copper–oxygen complex under turnover (Miller & Klinman, 1985; Kruse et al., 1986b). In contrast, binding of the multisubstrate inhibitor (I) causes a marked change in the copper ligand environment. The Cu edge of this sample (not shown) is slightly changed from the edge of ascorbate-reduced DBH but is still indicative of a low-coordinate Cu(I) site. Consistent with the design of this inhibitor (Kruse et al., 1986b), the presence of additional sulfur ligation is evident in the inhibited form of DBH relative to the ascorbate-reduced form (Figure 4b,e; Table II). On the basis of steady-state kinetic data, the multisubstrate inhibitor was previously suggested to bind directly to a copper atom via the sulfur. Our EXAFS data support this suggestion, further indicating that the inhibitor may be binding through the sulfur atom to only half the Cu(I) sites[3] (cf. coordination numbers of fit 11, Table II). It is intriguing that this is consistent with the inhibitor bridging the two Cu(I) sites of one subunit, coordinating through the sulfur to one Cu(I) and through the imidazole nitrogen to the other.

The Cu(II) form of DBH is inactive in the monooxygenase reaction catalyzed by this enzyme and is very possibly physiologically unimportant (Klinman & Brenner, 1988). Our results indicate that a major structural rearrangement of the copper active sites is at least partly responsible for the reductive activation of DBH. Further spectroscopic and physicochemical studies of the copper sites should be directed at the ascorbate-reduced form of DBH, since the Cu(II) sites in the oxidized enzyme have now been shown to be structurally distinct from the active Cu(I) sites.

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[3] We are grateful to a reviewer of this paper for suggesting the half-integer coordination number fits.

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Supplementary Material Available

Raw Cu XAS data for oxidized, ascorbate-reduced (two samples), ascorbate-reduced + tyramine, and ascorbate-reduced + multisubstrate inhibitor forms of dopamine β-hydroxylase (15 pages). Ordering information is given on any current masthead page.

Registry No. DBH, 9013-38-1; $[Cu(II)(Im)_4]^{2+}$, 47105-83-9; $[Cu(I)(BBDHp)](PF_6)_x(BF_4)_{1-x}$, 81372-13-6; $[Cu(I)(L_1\text{-}PR)](BF_4)$, 70814-12-9; Cu, 7440-50-8.

References

Ash, D. E., Papadopoulos, N. J., Colombo, G., & Villafranca, J. J. (1984) *J. Biol. Chem. 259*, 3395–3398.

Aunis, D., Miras-Portugal, M. T., & Mandel, P. (1975) *J. Neurochem. 24*, 423–431.

Baker, G. J., Knowles, P. F., Pandeya, K. B., & Rayner, J. B. (1986) *Biochem. J. 237*, 609–612.

Blumberg, W. E., Goldstein, M., Lauber, E., & Peisach, J. (1965) *Biochim. Biophys. Acta 99*, 187–190.

Co, M. S., Scott, R. A., & Hodgson, K. O. (1981) *J. Am. Chem. Soc. 103*, 986–988.

Dagdigian, J. V., McKee, V., & Reed, C. A. (1982) *Inorg. Chem. 21*, 1332–1342.

Friedman, S., & Kaufman, S. (1966) *J. Biol. Chem. 241*, 2256–2259.

Hall, D., & Waters, T. N. (1960) *J. Chem. Soc.*, 2644–2648.

Hasnain, S. S., Diakun, G. P., Knowles, P. F., Binsted, N., Garner, C. D., & Blackburn, N. J. (1984) *Biochem. J. 221*, 545–548.

Kahn, O., Galy, J., Journaux, Y., Jaud, J., & Morgenstern-Badarau, I. (1982) *J. Am. Chem. Soc. 104*, 2165–2176.

Karlin, K. D., Hayes, J. C., Gultneh, Y., Cruse, R. W., McKown, J. W., Hutchinson, J. P., & Zubieta, J. (1984) *J. Am. Chem. Soc. 106*, 2121–2128.

Kau, L.-S., Spira-Solomon, D. J., Penner-Hahn, J. E., Hodgson, K. O., & Solomon, E. I. (1987) *J. Am. Chem. Soc. 109*, 6433–6442.

Klinman, J. P., & Brenner, M. (1988) in *Fourth International Symposium on Oxidases and Related Redox Systems* (King, T. E., & Mason, H., Eds.) Liss, New York (in press).

Klinman, J. P., Krueger, M., Brenner, M., & Edmondson, D. E. (1984) *J. Biol. Chem. 259*, 3399–3402.

Knapp, S., Keenan, T. P., Zhang, X., Fikar, R., Potenza, J. A., & Schugar, H. J. (1987) *J. Am. Chem. Soc. 109*, 1882–1883.

Kruse, L. I., Kaiser, C., DeWolf, W. E., Jr., Frazee, J. S., Garvey, E., Hilbert, E. L., Faulkner, W. A., Flaim, K. E., Sawyer, J. L., & Berkowitz, B. A. (1986a) *J. Med. Chem. 29*, 2465–2472.

Kruse, L. I., DeWolf, W. E., Jr., Chambers, P. A., & Goodhart, P. J. (1986b) *Biochemistry 25*, 7271–7278.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Ljones, T., & Skotland, T. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 2, pp 131–157, CRC, Boca Raton, FL.

McKee, V., Zvagulis, M., Dagdigian, J. V., Patch, M. G., & Reed, C. A. (1984) *J. Am. Chem. Soc. 106*, 4765–4772.

Miller, S. M., & Klinman, J. P. (1985) *Biochemistry 24*, 2114–2127.

Nagatsu, T., & Udenfriend, S. (1972) *Clin. Chem. (Winston-Salem, N.C.) 18*, 980–983.

Obata, A., Tanaka, H., & Kawazura, H. (1987) *Biochemistry 26*, 4962–4968.

Preaux, G., & Gielens, C. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 2, pp 159–205, CRC, Boca Raton, FL.

Rosenberg, R. C., & Lovenberg, W. (1980) *Essays Neurochem. Neuropharmacol. 4*, 163–209.

Rush, R. A., Kindler, S. H., & Udenfriend, S. (1974) *Biochem. Biophys. Res. Commun. 61*, 38–44.

Schilstra, M. J., Birker, P. J. M. W. L., Verschoor, G. C., & Reedijk, J. (1982) *Inorg. Chem. 21*, 2637–2644.

Scott, R. A. (1982) in *The Biological Chemistry of Iron* (Dunford, H. B., Dolphin, D., Raymond, K. N., & Sieker, L., Eds.) pp 475–484, Reidel, Boston.

Scott, R. A. (1985) *Methods Enzymol. 117*, 414–459.

Scott, R. A., & Dooley, D. M. (1985) *J. Am. Chem. Soc. 107*, 4348–4350.

Scott, R. A., Schwartz, J. R., & Cramer, S. P. (1986) *Biochemistry 25*, 5546–5555.

Skotland, T., & Ljones, T. (1977) *Int. J. Pept. Protein Res. 10*, 311–314.

Skotland, T., & Ljones, T. (1979) *Inorg. Perspect. Biol. Med. 2*, 151–180.

Skotland, T., Petersson, L., Bäckström, D., Ljones, T., Flatmark, T., & Ehrenberg, A. (1980) *Eur. J. Biochem. 103*, 5–11.

Smith, T. A., Penner-Hahn, J. E., Berding, M. A., Doniach, S., & Hodgson, K. O. (1985) *J. Am. Chem. Soc. 107*, 5945–5955.

Solomon, E. I. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 41–108, Wiley, New York.

Teo, B.-K., & Lee, P. A. (1979) *J. Am. Chem. Soc. 101*, 2815–2832.

Teo, B.-K., Antonio, M. R., & Averill, B. A. (1983) *J. Am. Chem. Soc. 105*, 3751–3762.

Villafranca, J. J. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 263–289, Wiley, New York.

Walker, G. A., Kon, H., & Lovenberg, W. (1977) *Biochim. Biophys. Acta 482*, 309–322.

Weininger, M. S., Hunt, G. W., & Amma, E. L. (1972) *J. Chem. Soc., Chem. Commun.*, 1140–1141.

Wilcox, D. E., Porras, A. G., Hwang, Y. T., Lerch, K., Winkler, M. E., & Solomon, E. I. (1985) *J. Am. Chem. Soc. 107*, 4015–4027.