Preparation and Characterization of Half-Apo Dopamine-β-hydroxylase by Selective Removal of Cu_A. Identification of a Sulfur Ligand at the Dioxygen Binding Site by EXAFS and FTIR Spectroscopy

Brian J. Reedy and Ninian J. Blackburn*

Contribution from the Department of Chemistry, Biochemistry and Molecular Biology, The Oregon Graduate Institute of Science and Technology, P.O. Box 91000, Portland, Oregon 97291-1000

Received September 27, 1993®

Abstract: Progress has been made in determining the individual coordination of each of the copper sites (Cu_A and Cu_B) which comprise the active center in dopamine-β-hydroxylase. Previous studies (Blackburn et al. *J. Biol. Chem.* **1991**, *266*, 23 120–27) have determined the average ligand environment per copper in the fully metalated enzyme as two to three histidines and one to two O/N donors in the Cu(II) form changing to 2–3 histidines and 0.5 sulfur donors upon reduction to the Cu(I) form. Derivatives of the Cu(I) form of DBH have been made in which Cu_A has been selectively removed, allowing Cu_B, the O_2-binding center to be studied by EXAFS and FTIR. Cu_B has been found to be coordinated to two histidines (Cu–N = 1.99 ± 0.03 Å), a S-donor ligand (Cu–S = 2.25 ± 0.02 Å), and a fourth, as yet unidentified ligand X (Cu–X = 2.53 ± 0.03 Å). The FTIR spectrum of the carbonyl derivative of Cu_B indicates that ν_{CO} (2089 cm^{-1}) is identical to that found for the fully metalated enzyme, providing strong evidence that the Cu_B site is not perturbed by Cu_A removal. EXAFS results on Cu_B–CO indicate that CO binding does not displace the S ligand but appears to displace the weakly bound ligand X. These results provide the first evidence for the involvement of sulfur ligation at the dioxygen binding site of a copper monooxygenase. Amino acid residues which could act as potential S donors are discussed, and it is suggested that a methionine is the most likely candidate. The implications of sulfur ligation on the hydroxylation mechanism are discussed.

Dopamine-β-hydroxylase (DBH) catalyzes the hydroxylation of phenylethylamines and related substrates at the benzylic position. The enzyme is a copper-containing monooxygenase and is responsible for the conversion of dopamine to noradrenalin, a key step in the biosynthesis of catecholamine neurotransmitters. The catalytic mechanism of DBH is of particular interest due to overwhelming evidence that dioxygen binding and subsequent monooxygenase chemistry is carried out at mononuclear copper centers,[1] rather than the dicopper reaction chemistry which has been characterized for oxyhemocyanin,[2] tyrosinase,[3] and a number of inorganic model systems,[4] or the trinuclear clusters found in laccase,[5] ascorbate oxidase,[6] and particulate methane monooxygenase.[7] Magnetic resonance studies have identified the catalytic centers in oxidized DBH as a pair of mononuclear Cu(II) atoms separated by at least 6 Å.[8] Carbon monoxide, a competitive inhibitor with respect to O_2, binds to only one of the Cu(I) atoms in the reduced enzyme, thereby establishing structural and chemical inequivalence of the metal centers.[9,10] A further point of interest is the apparent difference in the average ligand environment (as determined by EXAFS) of the oxidized and reduced forms.[11-13] Thus the Cu(II) form of the enzyme appears to be ligated by an average of two to three histidines and one to two O donors per copper, whereas the Cu(I) form is ligated by two to three histidines and 0.5 sulfur donors per copper. Based on these observations and results from other laboratories,[14] we have recently proposed a model for the catalytic centers involving two structurally distinct Cu centers, one of which (Cu_B) acts as the dioxygen-binding and substrate hydroxylation site, while the

® Abstract published in *Advance ACS Abstracts*, February 1, 1994.

(1) (a) Blackburn, N. J. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman & Hall: New York, 1993; 164–183. (b) Stewart, L. C.; Klinman, J. P. *Annu. Rev. Biochem.* **1988**, *57*, 551–592.

(2) (a) Magnus, K. A.; Ton-That, H.; Carpenter, J. E. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman & Hall, New York, 1993; pp 143–150. (b) Solomon, E. I.; Baldwin, M. J.; Lowery, M. L. *Chem. Rev.* **1992**, 521–542. (c) Volbeda, A.; Hol, W. G. J. *J. Mol. Biol.* **1989**, *209*, 249–279.

(3) (a) Lerch, K. In *Metal Ions Biol. Syst.* **1981**, *13*, 143–186. (b) Robb, D. A. In *Copper Proteins and Copper Enzymes*; Lontie, R., Ed.; CRC Press: Boca Raion, FL, 1984; pp 207–241.

(4) (a) Karlin, K. D.; Gultneh, Y. *Prog. Inorg. Chem.* **1987**, *35*, 219–327. (b) Tyeklar, Z.; Karlin, K. D.; *Acc. Chem. Res.* **1989**, *22*, 241–248. (c) Kitajima, N. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman & Hall: New York, 1993; pp 251–263. (d) Kitajima, N.; Fujisawa, K.; Fujimoto, C.; Moro-oka, Y.; Hashimoto, S.; Kitagawa, T.; Toriumi, K.; Tatsumi, K.; Nakamura, A. *J. Am. Chem. Soc.* **1992**, *114*, 1277–1291. (e) Tyeklar, Z.; Jacobson, R. R.; Wei, N.; Murthy, N. N.; Zubieta, J.; Karlin, K. D. *J. Am. Chem. Soc.* **1993**, *115*, 2677–2689.

(5) (a) Allendorf, N. D.; Spira, D. J.; Solomon, E. I. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3063–3067. (b) Spira-Solomon, D. J.; Allendorf, M. D.; Solomon, E. I. *J. Am. Chem. Soc.* **1986**, *108*, 5318–5328. (c) Spira-Solomon, D. J.; Solomon, E. I. *J. Am. Chem. Soc.* **1987**, *109*, 6421–6432.

(6) (a) Messerschmidt, A.; Rossi, A.; Ladenstein, R.; Huber, R.; Bolognesi, M.; Gatti, G.; Marchesini, A.; Retruzelli, R.; Finazzi-Agro, A. *J. Mol. Biol.* **1989**, *206*, 513–529. (b) Messerschmidt, A. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman & Hall: New York, 1993; pp 471–484.

(7) (a) Chan, S. I.; Nguyen, H. T.; Shiemke, A.; K.; Lidstrom, M. E. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman & Hall: New York, 1993; pp 184–195.

(8) (a) Blackburn, N. J.; Collison, D.; Sutton, J.; Mabbs, F. E. *Biochem. J.* **1984**, *220*, 447–454. (b) Blackburn, N. J.; Concannon, M.; Khosrow Shahiyan, S.; Mabbs, F. E.; Collison, D. *Biochemistry* **1988**, *27*, 6001–6008. (c) McCracken, J.; Desai, P. R.; Papadopoulos, N. J.; Villafranca, J. J.; Peisach, J. *Biochemistry* **1988**, *27*, 4133–4137.

(9) Blackburn, N. J.; Pettingill, T. M.; Seagraves, K. S.; Shigeta, R. T. *J. Biol. Chem.* **1990**, *265*, 15383–15386.

(10) Pettingill, T. M.; Strange, R. W.; Blackburn, N. J. *J. Biol. Chem.* **1991**, *266*, 16996–17003.

(11) Blackburn, N. J.; Hasnain, S. S.; Pettingill, T. M.; Strange, R. W. *J. Biol. Chem.* **1991**, *266*, 23120–23127.

(12) Scott, R. A.; Sullivan, R. J.; De Wolfe, W. E.; Dolle, R. E.; Kruse, L. I. *Biochemistry* **1988**, *27*, 5411–5417.

(13) Blumberg, W. E.; Desai, P. R.; Powers, L.; Freedman, J. H.; Villafranca, J. J. *J. Biol. Chem.* **1989**, *264*, 6029–6032.

(14) (a) Stewart, L. C.; Klinman, J. P. *Biochemistry* **1987**, *26*, 5302–5309. (b) Brenner, M. C.; Murray, C. J.; Klinman, J. P. *Biochemistry* **1989**, *28*, 4656–4664. (c) Brenner, M. C.; Klinman, J. P. *Biochemistry* **1989**, *28*, 4664–4670.

0002-7863/94/1516-1924$04.50/0 © 1994 American Chemical Society

other (Cu_A) functions to shuttle electrons from the reducing cofactor, ascorbate, into this substrate hydroxylating site.[1a,9–11]

In order to understand the different catalytic roles of each copper implied by this model, it is essential to characterize the coordination chemistry of Cu_A and Cu_B individually. Furthermore, the mechanistic significance of the S donor is presently obscure, and it is of particular importance to establish whether the S is coordinated at Cu_A or at Cu_B, and whether its origin is a cysteine or a methionine residue. To address these questions we have developed methodologies to selectively remove one copper from each active site so that the other can be studied independently. In this paper we report our results on the selective removal of Cu_A from dopamine-β-hydroxylase, and our subsequent discovery that the S contribution increases in the EXAFS of this demetalated or "half-apo" derivative, establishing the location of the S donor at Cu_B, the dioxygen binding site.

Experimental Section

Enzyme Isolation and Assay. Dopamine-β-hydroxylase was isolated and assayed as previously described.[11] The specific activity of the fully metalated form of the enzyme was found to vary between 16 and 23 units/mg at 25 °C and atmospheric O_2. The protein concentration was estimated from the absorbance at 280 nm using a value of $A_{280}{}^{1\%} = 1.24$. The copper concentrations of all enzyme samples were determined using a Varian-Techtron flame atomic absorption spectrophotometer.

Measurement of CO–Binding Stoichiometry. The stoichiometry of CO binding to Cu in the fully metalated and half-apo derivatives was determined by spectrophotometric titration with deoxyhemoglobin, using a modification of a method first described by Zolla and Brunori.[15] This method makes use of the strong binding of CO to deoxyhemoglobin ($K_D \approx 50$ nM, R state) to quantitatively scavenge CO bound to Cu(I) centers in DBH ($K_D \approx 50$ μM).[9] A 13.5 μL sample of deoxyhemoglobin (about 2.75 mM in Fe) was prepared by reducing a concentrated Hb stock sample with a 30-times molar excess of ascorbate (0.33 M) under argon, in the presence of ascorbate oxidase (Sigma, 2.5 units). The ascorbate/ascorbate oxidase system was found to be extremely efficient for the preparation of analytically pure deoxyhemoglobin, since it both reduced the residual met-Hb and simultaneously ensured the complete removal of dissolved oxygen, and was found to be preferable to sodium dithionite (the reagent used in the original version of the procedure), which led to side reactions, and interfered with the spectrophotometric determination of CO bound to Hb. A 0.2-cm pathlength UV/vis cuvette, sealed with a rubber septum and containing 725 μL of an anaerobic solution of ascorbate (6.75 mM) and ascorbate oxidase (12.5 units) in acetate buffer (100 mM, pH 6.0), was prepared (under pure argon) and used to obtain a zero baseline on the UV/vis spectrophotometer (Shimadzu UV-265) in the 400–460 nm range. Using a Hamilton syringe, 8–10 μL of deoxyHb solution was injected into the cuvette to give an Hb concentration of about 0.03 mM in Fe. After mixing, this solution was allowed to stand until 100% reduction of the Hb was indicated by a single, stable Soret absorbance maximum at 430 nm. DBH-CO was prepared at 0 °C as described previously[9,10] in one chamber of an airtight double chamber apparatus, with the second chamber containing the identical buffer used in the preparation of the enzyme (50 mM potassium phosphate, pH 7.5). This ensured equilibration of the buffer and the enzyme with the same partial pressure of CO gas (1–1.3 atm). Aliquots (0.6 μL) of first the buffer, then the enzyme solution (0.5–1.5 mM in Cu), were added by syringe to the deoxyHb solution in the cuvette at room temperature, and the absorbance change at 419 nm (ΔA_{419}), the Soret maximum for HbCO, was measured for each aliquot. The Hamilton gas-tight syringe used in the titration was kept chilled to 0 °C when not in use, to ensure that CO did not come out of solution during aliquot transfers. Plots of ΔA_{419} versus volume added for each of the two solutions were linear (below saturation), indicating the quantitative transfer of $CO_{(buffer)}$ or $\{CO_{(DBH\text{-}CO)} + CO_{(buffer)}\}$ to the hemoglobin in the titration (Figure 1). The concentration of CO in each solution was then determined from the following equation, using the slope of the relevant plot

$$[CO] = \frac{\text{slope} \times (\text{vol Hb solution})}{(\epsilon_{HbCO} - \epsilon_{deoxyHb}) \times l}$$

where l is the cell pathlength, and the extinction coefficients shown are for 419 nm. (The difference between these coefficients is a constant, with an experimentally obtained value of 90 mM^{-1} cm^{-1}.) The concentration of enzyme-bound CO was given by the difference between the [CO] values for the two solutions.

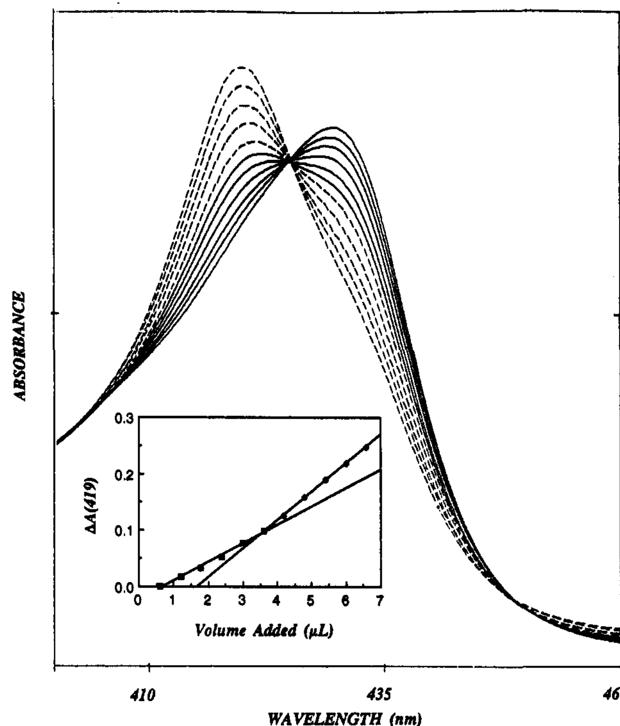


Figure 1. Spectrophotometric determination of enzyme-bound carbon monoxide, by conversion to the Hb–CO complex and measurement of the absorbance in the Soret region. Full lines correspond to additions of aliquots of CO-saturated buffer; dashed lines correspond to additions of aliquots of CO-saturated DBH in the same buffer. The inset shows the differences in the slope of the CO-saturated buffer and enzyme + buffer titration points, respectively.

Infrared Spectroscopy. Infrared spectra of DBH-CO samples were recorded at 10 °C as described previously,[10] on a Perkin-Elmer 1800 FTIR spectrometer, operating in transmission mode. The spectrometer's qualitative mode was chosen for spectral collection, giving a nominal resolution of 2 cm^{-1}. Reported spectra are differences of CO-bound and CO-free reduced enzyme.

EXAFS Spectroscopy. Three different "half-apo" DBH preparations, D, H, and J (see Table 1), were used in EXAFS experiments. Of these, sample D was obtained from the GF450XL column in half-apo form, while samples H and J were selectively demetalated by the cyanide dialysis method (*vide infra*). The latter two samples were concentrated from buffer containing 10–15% glycerol, to reduce the formation of the ice crystals upon freezing. The concentrated enzyme samples were transferred to small rectangular lucite cells (3.5 × 1.5 × 24 mm) having one side sealed with "Insulectro" mylar tape (25 μm in thickness, CHR Industries, New Haven, CT). Anaerobic transfers were achieved by placing the cell inside a small tube fitted with a septum and transferring the sample under an appropriate gas atmosphere via a syringe. The cell and sample were then immediately frozen in liquid nitrogen. Reduced, unligated EXAFS samples were frozen under pure argon; a carbonylated sample (from preparation H) was frozen under CO gas.

Measurement of X-ray Absorption Spectra. Samples were measured on beamline X9A at NSLS, Brookhaven National Laboratory, with an electron beam energy of 2.5 GeV and a maximum stored current of 220 mA. Data were collected with a Si(111) double crystal monochromator and a grazing incidence mirror to reject harmonics. Samples were measured as frozen glasses at 100 K in fluorescence mode using a 13-element Ge detector. A 3-μ Ni foil was placed immediately in front of the detector. Energy calibration was achieved by setting the first point of a copper foil spectrum to 8980.3 eV, and the resolution was estimated to be about 3 eV by inspection.

Raw data were averaged, background subtracted, and normalized to the smoothly varying background atomic absorption using standard procedures.[11] Data analysis was carried out by least-squares curve fitting utilizing full curved-wave multiple scattering calculations as formulated

(15) Zolla, L.; Brunori, M. *Anal. Biochem.* **1983**, *133*, 465–469.

by the SRS library program EXCURV[16-19] previously described.[1,11,20,21] The quality of the fits was determined using a least-squares fitting parameter, F, defined as

$$F^2 = (1/N)\sum k^6(\chi_i^{theor} - \chi_i^{exp})^2$$

and referred to as the fit index in subsequent discussion.

Results and Discussion

Preparation of selectively Demetalated (Half-Apo) DBH. EPR and EXAFS data on dopamine-β-hydroxylase indicate that the Cu(II) form of the enzyme appears to be ligated by an average of two to three histidines and one to two O donors per copper,[1,8,11] whereas the Cu(I) form is ligated by two histidines and 0.5 sulfur donors per copper.[1,10-13] Furthermore, recent work from our laboratory has established that the copper centers in the reduced enzyme are chemically inequivalent.[9,10] The inequivalence derives from the discovery that CO, a competitive inhibitor with respect to O_2, only binds to one of the Cu(I) centers in the active site, and this observation forms the basis for our proposed model for the catalytic centers involving two structurally distinct Cu centers, Cu_A and Cu_B; Cu_B acts as the dioxygen-binding and substrate hydroxylation site, while Cu_A functions to shuttle electrons from the reducing cofactor, ascorbate, into this substrate hydroxylating site. Delineation of the individual catalytic roles of Cu_A and Cu_B clearly requires the selective removal of one copper so that the coordination chemistry and reactivity of the other can be studied individually. However, despite the chemical and functional differences between Cu_A and Cu_B, the formation constants for Cu(II) binding are similar and unusually low for a copper enzyme.[8b] This combination has made it impossible to achieve selective demetalation using the more traditional methods of selective removal of Cu(II) by chelating agents with pK_m values between those of Cu_A and Cu_B.

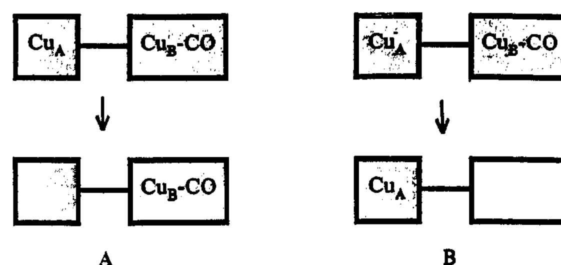
Notwithstanding these difficulties, we have achieved selective removal of Cu_A by two independent methods. The first method consisted simply of observing that enzyme obtained fresh from the GF450XL gel-filtration column, (the final stage in the preparation) was frequently in a "demetalated" form (2.5–4 Cu per tetramer), which analyzed (*vide infra*) as containing pure Cu_B. However, this method subsequently proved unreliable, prompting us to develop the systematic procedure described below, which makes use of the competition between CO and CN^- for the Cu(I) centers. CN^- interacts with both oxidized and reduced DBH removing both copper centers as Cu(I)–cyano complexes.[8a,22] Recent studies of speciation in CN-treated solutions of Cu proteins and model compounds suggests that the mechanism of copper removal involves the formation of protein-bound Cu(II)– and Cu(I)–cyano complexes as obligatory intermediates on route to the formation of the thermodynamically favored tricyanocuprate and tetracyanocuprate complexes.[23] We reasoned therefore that since CO binds selectively to one Cu center, it should compete with cyanide for this site and protect it from copper loss. As a consequence, Cu_A should be selectively removed.

The protocol for producing Cu_A-depleted DBH from fully metalated enzyme was as follows. The semidemetalated enzyme isolated from HPLC gel-filtration was reconstituted with Cu(II) by addition of a slight molar excess of cupric nitrate and washing away the excess Cu(II) by ultrafiltration. Samples with Cu concentrations of about 200–500 μM were dialyzed for several hours against CO-saturated anaerobic buffer, reduced with a stoichiometric amount of ascorbate, and then allowed to dialyze overnight against a CO-saturated buffer solution of NaCN (25 μM) and NaCl (50 μM). The protein was then washed in fresh CO-saturated anaerobic buffer to remove CN^- and cyano-copper species. The consistent product of this procedure was DBH containing 2.2–3.8 Cu per tetramer, independent of the cyanide dialysis time (6–36 h).

In order to show that the product was indeed selectively demetalated or half-apo, we carried out CO-binding assays. As illustrated in Scheme 1, selective removal of Cu_A is expected to produce a derivative which binds one CO per copper, while selective removal of Cu_B should produce a derivative which does not bind CO. The third case of statistical removal of copper from each site should produce a derivative which retained the CO-binding stoichiometry of the native reduced enzyme, namely 0.5 CO per copper.

The results of the CO-binding analysis of ten independent preparations of the half-apo enzyme are shown in Table 1. Samples A–D were demetalated samples analyzed immediately after elution from the gel-filtration column, whereas samples E–K were prepared by the cyanide dialysis method described above. It can be seen that considerable variation in Cu/protein binding stoichiometry is present, ranging between 3.8 for preparation G and 2.2 for preparation B. Also, the total copper concentration varies over a 4-fold range (0.3–1.1 mM). However, in all samples listed, selective removal of Cu_A is demonstrated by

Scheme 1



CASE A: *Binds 1 CO per Copper*

CASE B: *Does not bind CO*

Table 1. Copper-Binding Stoichiometries and Carbon Monoxide-Binding Ratios for Ten Independent Preparations of Selectively Demetalated Dopamine-β-hydroxylase

sample	[Cu]/ mM	[Cu]/ [DBH] tetramer	[CO]/ [Cu][a] off GF450	[CO]/ [Cu][b] CN⁻ treated	[CO]/ [Cu][c] reconstituted
A	0.86	3.4	1.08		0.45
B	1.12	2.2	1.02		0.56
C	0.63	2.7	1.03		0.42
D[d]	0.72	2.7	0.94		
E	0.31	2.3		1.05	
F	0.45	2.6		1.07	
G	0.74	3.8	0.52	1.06	
H[d]	0.97	3.6	0.77	0.85	
J[d]	0.51	2.7		1.04	
K	0.63	3.3		0.96	

[a] This column represents the [CO]/[Cu] ratios measured immediately after elution from the GF450XL HPLC gel-filtration column, the final step in the purification procedure. [b] This column represents the [CO]/[Cu] ratios measured after preparation of the selectively demetalated samples by the cyanide dialysis method. [c] This column represents the [CO]/[Cu] ratios measured after reconstitution of the demetalated samples to their full complement of copper. [d] Samples studied by EXAFS.

(16) Binsted, N.; Gurman, S. J.; Campbell, J. W. SERC Daresbury Laboratory EXCURV88 Program; 1988.
(17) Gurman, S. J. In *Synchrotron Radiation and Biophysics*; Hasnain, S. S., Ed.; Ellis Horwood Ltd.: Chichester, United Kingdom, 1989; pp 9–42.
(18) Gurman, S. J.; Binsted, N.; Ross, I. *J. Phys. C* **1984**, *17*, 143–151.
(19) Gurman, S. J.; Binsted, N.; Ross, I. *J. Phys. C.* **1986**, *19*, 1845–1861.
(20) Strange, R. W.; Blackburn, N. J.; Knowles, P. F.; Hasnain, S. S. *J. Am. Chem. Soc.* **1987**, *109*, 7157–7162.
(21) Strange, R. W.; Hasnain, S. S. In *Synchrotron Radiation and Biophysics*; Hasnain, S. S., Ed.; Ellis Horwood Ltd.: Chichester, United Kingdom, 1989; pp 105–121.
(22) Obata, A.; Tanaka, H.; Kawazura, H. *Biochemistry* **1987**, *26*, 4962–4968.
(23) Han, J.; Blackburn, N. J.; Loehr, T. M. *Inorg. Chem.* **1992**, *31*, 3223–3229.

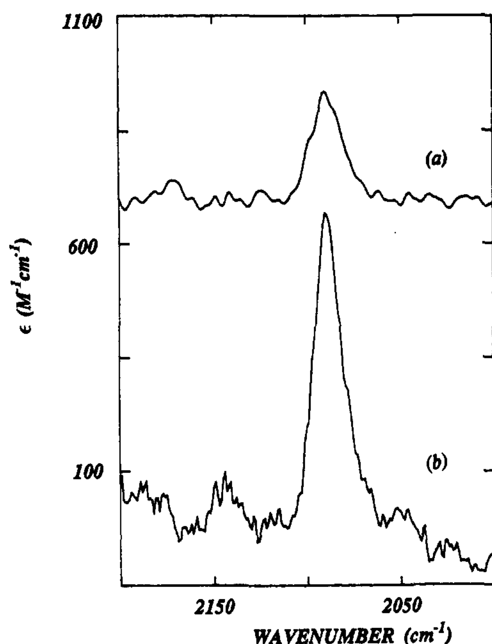


Figure 2. FTIR spectra in the CO intraligand stretching region of (a) half-apo (Cu_B–CO) and (b) fully metalated (Cu_A + Cu_B–CO)DBH.

a CO-binding stoichiometry of close to 1.0. As stated above, only the cyanide dialysis method was found to give consistent results, since analysis of preparations immediately after elution from gel-filtration gave results which ranged from selective (A–D) to statistical demetalation (G) (Table 1). Analysis of preparations E, F, J, and K was only performed after the cyanide dialysis procedure.

As a control, samples A–C were reconstituted with Cu(II) to the fully metalated forms (eight Cu per tetramer) and then subjected to CO-binding analysis. The results shown in the last column of Table 1 demonstrate that the high CO-binding ratios found immediately after isolation do not arise from active sites with modified CO-binding chemistry but are indeed genuine half-apo derivatives as isolated, since they can be reconstituted to fully-metalated forms with normal CO-binding characteristics. It was also observed that in most cases, the half-apo derivatives slowly reverted to statistical loading of metal centers unless stored under a high (>1 atm) partial pressure of CO.

Selective metal removal from active sites in proteins can potentially produce perturbations in the structure of the remaining metal center(s). In order to address this question we have compared the FTIR spectrum of the carbon monoxide complexes of half-apo proteins prepared by both methodologies. The results are shown in Figure 2. ν(CO) of the half-apo DBH is reproducible and occurs at 2089 cm^{-1}. This is the identical frequency to that found previously for fully metalated enzyme.[10] The C≡O stretching frequency in Cu(I) carbonyls is very sensitive to small changes in the Cu(I) environment,[1,4d,9,24] and substrate binding to the fully metalated enzyme causes a 3-cm^{-1} shift to lower energy.[10] The extreme sensitivity of ν_{CO} to local environment and in particular to interactions of the metal-bound CO with noncoordinated residues is well illustrated by recent studies on mutant CO–myoglobins (Mb–CO), in which mutations of the distal residues His-64 and Val-68 produce large shifts in ν_{CO}: these shifts have been assigned to changes in the magnitude of the Fe–C–O angle and/or to H-bonding or electrostatic field effects on the O atom of the carbonyl group, both of which can modulate the magnitude of the π-backbonding from Fe^{2+} to CO.[25,26] The coincidence in the ν_{CO} of half-apo and fully metalated DBH thus suggests that perturbations in the Cu_B site induced by Cu_A removal are minimal and validates the interpretation of EXAFS data on the half-apo enzyme described below, as truly representative of the intact dioxygen-binding Cu_B site. On the other hand, the intensity of the IR band is substantially diminished. The molar extinction coefficient calculated per mol of bound CO is approximately one-third of that of the fully metalated CO derivative. Since the intensity is proportional to the magnitude of the C–O dipole moment, it would appear that removal of Cu_A decreases the electric dipole oscillator strength. If the C → O dipole were oriented in the direction of Cu_A, then removal of the latter might eliminate the component of charge polarization from the electrostatic field of the Cu_A^+, provided Cu_A were in reasonable proximity to Cu_B. However, the linear correlation between C–^{17}O chemical shifts in the ^{17}O NMR spectrum and ν_{CO} of a large number of distal-side mutant heme carbonyls has clearly shown that changes in the electrostatic environment are expected to cause changes in frequency as well as intensity,[26] rendering this explanation overly simplistic. Disruption of charge relay or H-bond networks by metal removal are also potential mechanisms for intensity modulation, but further work is needed before a convincing explanation for the reduction in extinction of the 2089 cm^{-1} half-apo DBH-CO band can be advanced.

EXAFS data have been measured on three independent preparations of ascorbate-reduced half-apo enzyme, corresponding to D, H, and J in Table 1. As discussed above, sample D was prepared by ascorbate reduction of the enzyme immediately after chromatographic elution. However samples H and J were initially prepared as their carbonylated forms, since they represent the product of cyanide dialysis of ascorbate-reduced enzyme under a CO atmosphere. Accordingly, samples H and J were decarbonylated by repeated vacuum flushing with pure argon. All three samples gave similar background-subtracted EXAFS spectra and confirmed that the same product was reproducibly obtained by either method.

Figure 3 compares the EXAFS of fully metalated DBH (Figure 3a) with that of the selectively demetalated enzyme isolated directly from gel-filtration (sample D, Figure 3b) or by the cyanide dialysis method (sample H, Figure 3c). It is evident that substantial changes accompany Cu_A removal. In particular, the amplitudes of the demetalated spectra appear considerably higher. It can be seen from inspection of Figure 3a,c that the first shell in the transform is split into two components at *ca.* 1.9 and 2.3 Å, respectively, but that while the amplitude of the 1.9 Å component remains approximately constant, that of the 2.3 Å component increases by *ca.* a factor of 2 in the half-apo spectrum, Figure 3c. Previous studies of the fully-metalated enzyme by us and others concluded that the 2.3 Å component was derived from an endogenous sulfur donor ligand but could not establish whether it was coordinated at Cu_A or Cu_B.[11,12] The analysis of the fully-metalated data from this laboratory showed conclusively that the second-shell scatterer was a S, not a Cl. A number of different lines of evidence (described in detail in ref 11) were used to arrive at this conclusion and included the observations that the EXAFS was invariant in the presence of added bromide ions even though Cu(I) is expected to form stronger complexes with Br than Cl. Identical EXAFS was also observed when the enzyme was isolated under conditions which rigorously excluded chloride and which gave preparations with analytical concentrations of chloride less than 0.1 Cl atoms per Cu. Best fits were obtained from an

(24) (a) Pasquali, M.; Floriani, C. In *Copper Coordination Chemistry, Biochemical and Inorganic Perspectives*; Karlin, K. D., Zubieta, J., Eds.; Adenine Press: New York, 1984; pp 311–330. (b) Villacorta, G. M.; Lippard, S. J. *Inorg. Chem.* **1987**, *26*, 3672–3676. (c) Sorrell, T. N.; Borovick, A. S. *J. Am. Chem. Soc.* **1986**, *108*, 2479–2481. (d) Sorrell, T. N.; Borovick, A. S. *J. Am. Chem. Soc.* **1987**, *109*, 4255–4260. (e) Sorrell, T. N.; Malachowski, M. R. *Inorg. Chem.* **1983**, *22*, 1883–1887. (f) Patch, M. G.; Choi, H.; Chapman, D. R.; Bau, R.; McKee, V.; Reed, C. A. *Inorg. Chem.* **1990**, *29*, 110–119. (g) Thompson, J. S.; Whitney, J. F. *Inorg. Chem.* **1984**, *23*, 2813–2819.

(25) Balasubramanian, S.; Lambright, D. G.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4718–4722.

(26) Park, K. D.; Guo, K.; Adebodun, F.; Chiu, M. L.; Sligar, S. G.; Oldfield, E. *Biochemistry* **1991**, *30*, 2333–2347.

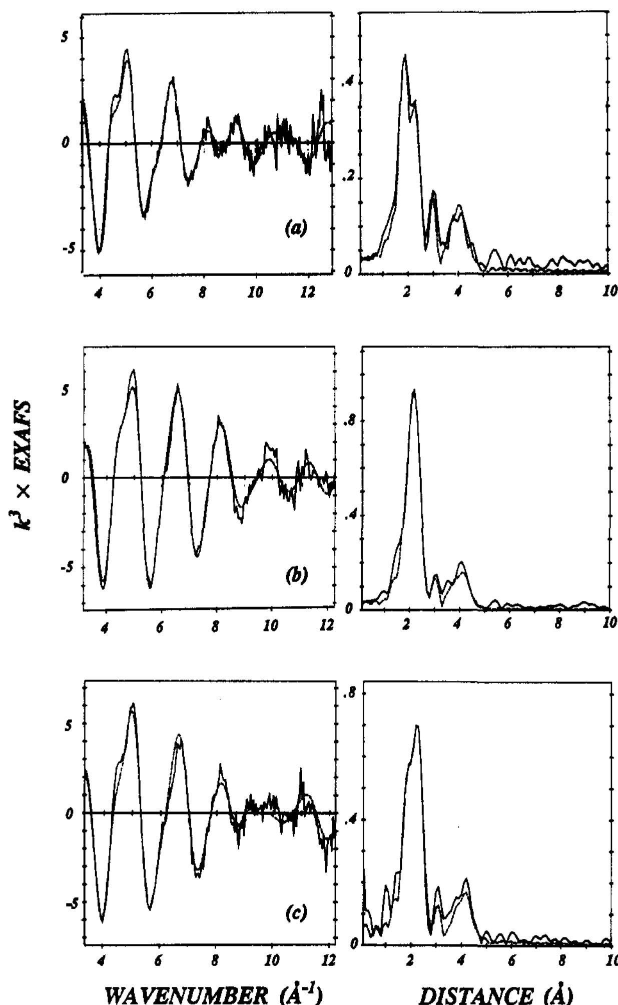


Figure 3. Experimental (solid) versus simulated (dashed) EXAFS and Fourier transforms for half-apo and fully metalated DBH: (a) fully metalated enzyme, (b) half-apo, sample D, and (c) half-apo sample H. Full parameter sets relating to the simulations are available as supplementary material (Tables S1–S3).

occupation number of 0.5 S per copper, suggesting that the S ligand was bound at only one of the copper centers. We speculated that the most likely locus was at Cu_A, since this was presumed to be the site of electron entry into the protein, and was thus analogous to the blue (His_2-Cys-Met) electron-transfer sites of multicopper enzymes such as ascorbate oxidase[6] and nitrite reductase.[27]

The present study indicates that, contrary to these initial speculations, the S donor is located at Cu_B. The EXAFS of both demetalated derivatives was simulated by least-squares curve fitting as follows. An initial parameter set was chosen with coordination numbers and bond lengths fixed at the best-fit values for the fully metalated protein, namely two histidines at 1.93 Å, 0.5 sulfurs at 2.23 Å, and a fourth low-Z ligand X, at 2.56 Å. These parameters were then floated in the least-squares fitting procedure. The refinement converged rapidly to two histidines, one sulfur, and the fourth ligand X at the distances given in Table 2. The data show that the differences between the EXAFS of fully-metalated and half-apo enzymes can be interpreted almost entirely by an increase in the sulfur component from an average of 0.5 S per Cu in fully-metalated to one S per Cu in half-apo. This implies that the sulfur ligand is bound exclusively at the Cu_B site.

The bond lengths and Debye–Waller factors for all three

(27) (a) Gooden, J. W.; Turley, S.; Teller, D. C.; Adman, E. T.; Liu, M. Y.; Payne, W. J.; LeGall, P. J. *Science* **1991**, *253*, 438–442. (b) Adman, E. T. *Adv. Protein Chem.* **1991**, *42*, 145–197.

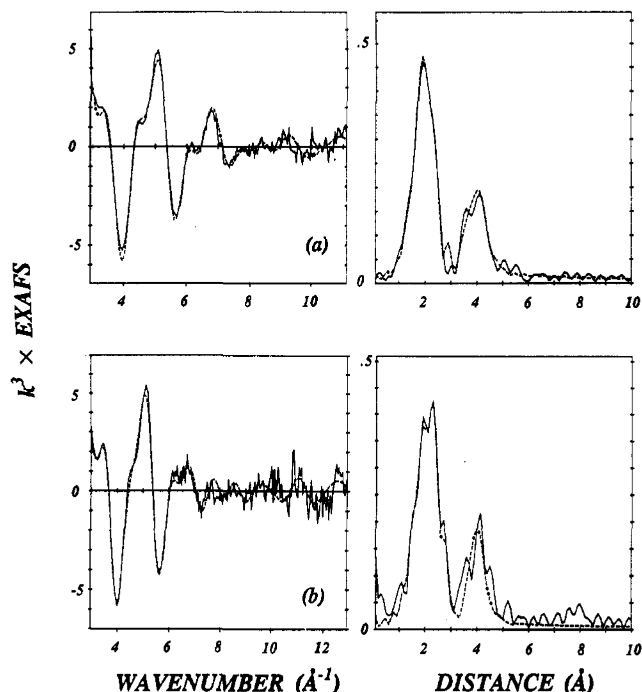


Figure 4. Experimental (solid) versus simulated (dashed) EXAFS and Fourier transforms for the carbonyl complexes of half-apo and fully metalated DBH: (a) fully metalated enzyme and (b) half-apo, sample H. Full parameter sets relating to the simulations are available as supplementary material (Tables S5 and S6).

samples of demetalated enzyme are similar but not identical. The differences almost certainly reflect errors introduced by differences in signal-to-noise. Thus while the coordination numbers and Cu–S bond lengths for all three samples are within the usual limits of precision (±25% for coordination numbers, ±0.02 Å for R_{Cu-S}), the Cu–N(imid) bond length has a slightly higher spread, *viz.* 1.96 Å in H and 2.00 Å in D and J. A mean value of Cu–N(imid) = 1.99 ± 0.03 Å can be extracted from this data. The average value of the Cu–N(imid) bond lengths at Cu_B is therefore 0.06 Å longer than the average of Cu–N(imid) for Cu_A + Cu_B, indicating that Cu–N(imid) at Cu_A is probably shorter than 1.93 Å. The value of Cu–N = 1.99 Å is consistent with the distorted four-coordination for Cu_B suggested from the EXAFS analysis, whereas a value of Cu–N < 1.93 Å would imply three-coordination or lower at Cu_A.[28] The latter inference is perhaps to be expected on the grounds that the less tightly bound metal ion is likely to be selectively removed.

Figure 4 compares EXAFS spectra of the carbonyl derivatives of fully-metalated (Figure 4a) and demetalated sample H (Figure 4b). The differences between the spectra are less obvious, especially since both spectra show very little EXAFS in the k = 7–10 Å^{-1}, due to destructive beating between CO multiple scattering and S waves in this region. This effectively reduces the data range and puts theoretical limits on the precision of the simulation parameters. Nevertheless, we have been able to show that the EXAFS of the half-apo carbonyl is consistent with CO binding to a Cu_B site composed of two His and one S ligand as

(28) (a) Three-coordinate Cu(I) complexes with N donor ligands typically have Cu–N bond lengths between 1.94 and 1.90 Å, although one Cu–N bond is often longer than the other two, giving rise to distorted T-shaped structures. Two-coordinate complexes typically have Cu–N below 1.90 Å. For reviews which describe structural data on Cu(I) complexes see, for example, ref 4 and the following: (b) Sanyal, I.; Karlin, K. D.; Strange, R. W.; Blackburn, N. J. *J. Am. Chem. Soc.* **1993**, *115*, 11 259–11 270. (c) Vigato, P. A.; Tamburini, S.; Fenton, D. E. *Coord. Chem. Rev.* **1990**, *106*, 25–170. (d) Blackburn, N. J.; Strange, R. W.; Reedijk, J.; Volbeda, A.; Farook, A.; Karlin, K. D.; Zubieta, J. *Inorg. Chem.* **1989**, *28*, 1349–135. (e) Kau, L.; Spira-Solomon, D. J.; Penner-Hahn, J. E.; Hodgson, K. O.; Solomon, E. I. *J. Am. Chem. Soc.* **1987**, *109*, 6433–6422. (f) Sorrell, T. N.; Garrity, M. L.; Richards, J. L.; Pigge, C.; Allen, W. E. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman & Hall: New York, 1993; pp 338–347.

Table 2. Metrical Parameters Used in the Simulation of the EXAFS Spectra of Selectively Demetalated Dopamine-β-hydroxylase Samples D, H, and J, Compared with Those of the Fully Metalated Enzyme

fully metalated			selectively demetalated (D)		
shell	distance (Å)	Debye–Waller (Å²)	shell	distance (Å)	Debye–Waller (Å²)
2 N (His)[a]	1.93	0.010	2 N (His)	2.00	0.007
0.5 S	2.25	0.006	1.0 S	2.25	0.003
1 O/N	2.56	0.012	1 0/N	2.53	0.009
selectively demetalated H			**selectively demetalated J**		
shell	distance (Å)	Debye–Waller (Å²)	shell	distance (Å)	Debye–Waller (Å²)
2 N (His)	1.96	0.006	2 N (His)	2.00	0.013
1.0 S	2.25	0.004	1.18 S	2.25	0.003
1 O/N	2.53	0.009	1 O/N	2.54	0.009

[a] The histidine rings were simulated as geometrically rigid units, using the multiple scattering protocols of the program EXCURV, as described under methods. Thus, the fits all contain outer shell C and N atoms at the appropriate positions for an imidazole ligand with Cu–N bond lengths as given in the table. Full details of the imidazole ring geometrical parameters are given in the supplementary material.

suggested above. Inspection of the Fourier transforms shows that, similar to the noncarbonylated systems, the 2.3 Å shoulder seen in the fully metalated enzyme has now become the dominant peak in the first shell. In addition the resolved feature in the EXAFS at *ca.* $k = 3.5$ Å^{-1}, which is associated with CO multiple scattering, is more intense in the demetalated CO derivative, in line with the increase in CO:Cu binding stoichiometry.

The results of simulation of the CO data are shown in Table 3. Because of the limited range of data with high S/N, we have fixed the CO occupation number at the experimentally determined value for this sample (H, Table 1) which is 0.85 per Cu. Simulations showed that because of the correlation between S occupation number and Debye–Waller factor, the fit would tolerate S coordination numbers in the range 0.6–1.1 with Debye–Waller factors of 0.005–015 Å², respectively.[29] The fit shown in Figure 4b is for 0.85 S per Cu, i.e., one S per CO-binding site, together with 0.85 CO and 2.0 His. The quality of the fit is good, and all the features in the EXAFS (including the rather poorly resolved and noisy features above $k = 7$ Å^{-1}) are well reproduced. Thus we have confidence that the CO derivative of the demetalated (Cu_B) site is consistent with a $Cu(His)_2S(CO)$ structure.

The bond lengths for Cu_B–CO are essentially unchanged from those found in the fully metalated carbonyl. However, the Debye–Waller terms are somewhat larger, and comparison with Table 2 indicates that there is a trend toward increasing DW terms in the CO derivatives over the noncarbonylated enzymes for both fully metalated and demetalated enzymes. Thus it would appear that the Cu_B site is more conformationally mobile in the carbonylated state than in the nonligated state. We have no explanation for this empirical observation, but note that conformational flexibility may assist correct alignment of the substrate with bound O_2 in the active site pocket. As found previously for the fully metalated carbonyl,[10] the simulations give no indication of the presence of the ligand X and suggest that this, weakly bound (O/N) group is displaced when CO (or O_2) binds within the active-site pocket. It is known from studies using mechanism-based inhibitors that two tyrosines (Tyr-216 and Tyr-477) are in close proximity to the active site and thus may be candidates for the identity of X.[30,31]

The finding that the O_2/CO binding site of dopamine-β-hydroxylase contains a sulfur ligand is unexpected on the basis of our present understanding of copper-dioxygen chemistry. A number of well-characterized copper–dioxygen complexes exist, and all of these can be formulated as copper(II)–peroxo (or superoxo) species with oxygen or nitrogen donor coordination.[4] The crystal structure of oxyhemocyanin from *Limulus* reveals an equatorial η^2:η^2-peroxo group coordinated in a coplanar fashion between the metal atoms of the dicopper unit, with two additional equatorial and one axial histidine bound to each tetragonal Cu(II).[2a] Both the structure and the spectroscopy of oxyhemocyanin are accurately mimicked by $[HB(2,3\text{-}iPr_2\text{-}pz)_3Cu]_2O_2$,[4c,d] which also contains the planar η^2:η^2-peroxo–dicopper unit with three substituted pyrazole ligands coordinated at each copper. Other modes of dioxygen binding have been characterized, such as the *trans*-1,2-peroxo bridge found in $[TMPA\text{-}Cu]_2O_2^{2+}$, in which the peroxo ligand occupies an equatorial position at each essentially trigonal-bipyramidal Cu(II) center, the remaining coordination positions being occupied by N atoms from pyridines and the tertiary amine group.[4e] More recently, a copper–dioxygen complex containing only unidentate 1,2-dimethylimidazole ligands has been described spectroscopically.[28b] This system is interesting in that the linear two-coordinate complex $[Me_2im]_2Cu^+$ does not react with either O_2 or CO, whereas addition of 1 additional equivalent of Me_2im per Cu(I) generates a distorted three-coordinate complex which is extremely sensitive to O_2 and forms a dinuclear dioxygen adduct formulated as $[\{Me_2im\}_3Cu]_2O_2^{2+}$ at or below −90 °C as well as a mononuclear carbonyl ($[(Me_2im)_3CuCO]^+$). The mode of dioxygen binding in the former is uncertain, but either end-on or "bent-butterfly" side-on structures were found to be consistent with the EXAFS data.[28b,32]

Given the preponderance for N-heterocyclic ligands in these copper–dioxygen structures, the mechanistic basis for an S donor at the dioxygen-binding site of DBH is intriguing. One important difference exists between dopamine-β-hydroxylase and hemocyanin and the model systems discussed above, namely the nuclearity of the copper centers. Thus, whereas oxyHc and all of the models are dinuclear, Cu_B in DBH is most certainly mononuclear. As suggested recently by Klinman,[33] O–O bond fission and substrate hydroxylation may require the generation of a copper(II)–oxygen radical intermediate ($Cu(II)O^•$) in which the oxidizing equivalent can be delocalized over only one copper and may represent a rationale for the presence of a stronger electron donor (sulfur) at the Cu_B site. However, as discussed below, only thiolate appears to be a sufficiently strong donor to fulfill such a role, and there are strong arguments against cysteine coordination. The other main candidate, methionine, appears to be a weaker donor than imidazole. These considerations emphasize the importance of determining the origin of the S donor (thiol, thiolate, thioether, or disulfide) before any conclusions as to its mechanistic role can be inferred.

Carbonyl complexes of Cu(I) coordinated by three N-heterocyclic donor groups typically have ν_{CO} between 2065 and 2085 cm^{-1}.[1a,24] We have argued that the value of 2089 cm^{-1} found for the Cu_B–CO complex is above the range expected for a $Cu(his)_3$-CO complex, and it would seem likely tht the increase in ν_{CO} results from the presence of the S-donor ligand. Methionine coordination would produce such an increase, since the poorer donor properties of a thioether ligand should reduce the π-back-

(29) Contour diagrams representing the surface mapped out by the fit index as coordination numbers and Debye–Waller factors are systematically varied and are provided in the supplementary material.

(30) Farrington, G. C.; Kumar, A.; Villafranca, J. J. *J. Biol. Chem.* **1990**, *265*, 1036–1040.

(31) De Wolfe, W. E.; Carr, S. A.; Varrichio, A.; Goodhart, P. J.; Mentzer, M. A.; Roberts, G. D.; Southan, C.; Dolle, R. E.; Kruse, L. I. *Biochemistry* **1988**, *27*, 9093–9101.

(32) Sanyal, I.; Strange, R. W.; Blackburn, N. J.; Karlin, K. D. *J. Am. Chem. Soc.* **1991**, *113*, 4692–4693.

(33) Klinman, J. P.; Berry, J. A.; Tian, G. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman & Hall: New York, 1993; pp 151–163. (b) Tian, G.; Klinman, J. P. *J. Am. Chem. Soc.* **1993**, *115*, 8891–8897. (c) Tian, G.; Berry, J. A.; Klinman, J. P. *Biochemistry* **1994**, *33*, 226–234.

bonding from Cu(I) to CO.[24e] On the basis of a highly conserved[34] HHM motif found in all DBH sequences[35] and in the sequences[36] of the mechanistically similar peptide amidating enzyme (PHM),[37] we have suggested[10,11] that the flanking H and M of this HHM motif formed the ligands to one of the coppers, while the middle histidine coordinated to the other copper, thereby establishing a connectivity pattern similar to that observed between copper centers in ascorbate oxidase and nitrite reductase.[6,10,11,27] However, recent unpublished results obtained in collaboration with R. E. Mains and B. A. Eipper that mutagenesis of the corresponding Met-109 to isoleucine in PHM generates an active mutant enzyme, appear to argue against this particular methionine (DBH Met-251) as the origin of the coordinated S. One other conserved methionine Met-486 in DBH, Met-314 in PHM is found in the homologous catalytic domain.[34] DBH Met-486 now appears to be a strong candidate for the sulfur ligand to Cu_B, particularly since it is in close proximity to Tyr-477 which is modified by the mechanism-based inhibitors 6-hydroxybenzofuran and phenylhydrazine, and thus must be located near the substrate binding site. It is significant that both Tyr-477 and Met-486 are both conserved between DBH and PHM.[34]

The reactivity of a coordinated thioether ligand in a copper monooxygenase model system has been investigated by Casella et al.[38] Their model system involves the hydroxylation of the bridging xylyl moiety in a dicopper(I) model complex containing nonlinear two-coordinate Cu(I) atoms. Substitution of the *N*-MeIm or py ligand at each Cu(I) by methyl–ethyl–thioether caused a 30-fold decrease in the rate of ring hydroxylation. This decrease in rate was ascribed to stabilization of the Cu(I) state by the thioether ligand. The relevance of this result to the mechanism of DBH is not clear at the present time, since no detailed kinetic or thermodynamic information is available on Casella's model system, but the study does not provide a strong argument for a significant catalytic advantage accruing from methionine coordination at Cu_B in dopamine-β-hydroxylase.

Turning now to the possibility of thiolate coordination, there are six conserved cysteine residues in the homologous catalytic domains of DBH and PHM.[34] It is likely that most or all of these are involved in disulfide bridge formation. In addition, other spectroscopic considerations argue powerfully against thiolate being the origin of the S-donor. Myoglobin and cytochrome-P450 both contain the protoporphyrin IX group but the proximal ligand in myoglobin is histidine, whereas in P450 it is thiolate. Above pH 7, ν_{CO} of the major component of Mb–CO is found

(34) Southan, C.; Kruse, L. I. *FEBS Lett.* **1989**, *255*, 116–120.
(35) (a) Lamouroux, A.; Vigny, A.; Faucon-Biguet, N.; Darmon, M. C.; Franck, R.; Henry, J. P.; Mallet, J. *EMBO J.* **1987**, *6*, 3931–3937. (b) Wang, N.; Southan, C.; De Wolfe, W. E.; Wells, T. N. C.; Kruse, L. I.; Leatherbarrow, R. J. *Biochemistry* **1990**, *29*, 6466–6474. (c) Taljanidisz, J.; Stewart, L.; Smith, A. J.; Klinman, J. P. *Biochemistry* **1989**, *28*, 10054–10061. (d) Lewis, E. J.; Allison, S.; Fader, D.; Claflin, V.; Baizer, L. *J. Biol. Chem.* **1990**, *265*, 1021–1028. (e) Mcmahon, A.; Geertman, R.; Sabban, E. L. *J. Neurosci. Res.* **1990**, *25*, 395–404. (f) Robertson, J. G.; Desai, P. R.; Kumar, A.; Farrington, G. K.; Fitzpatrick, P. F.; Villafranca, J. J. *J. Biol. Chem.* **1990**, *265*, 1029–1035.
(36) (a) Stoffers, D. A.; Barthel-Rosa Green, C.; Eipper, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 735–739. (b) Stoffers, D. A.; Ouafik, L.; Eipper, B. A. *J. Biol. Chem.* **1991**, *266*, 1701–1707. (c) Eipper, B. A.; Perkins, S. N.; Husten, E. J.; Johnson, R. C.; Keutmann, H. T.; Mains, R. E. *J. Biol. Chem.* **1991**, *266*, 7827–7833.
(37) (a) Eipper, B. A.; Mains, R. E.; Glembotski, C. C.; *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5144–5148. (b) Young, S. D.; Tamburini, P. P. *J. Am. Chem. Soc.* **1989**, *111*, 1933–1934. (c) Noguchi, M.; Seino, H.; Kochi, H.; Okomoto, H.; Tanaka, T. *Biochem. J.* **1992**, *283*, 883–888. (d) Kawahara, T.; Suzuki, K.; Iwasaki, Y.; Shimoi, H.; Akita, M.; Moro-oka, Y.; Nisikawa, Y. *J. Chem. Soc., Chem. Commun.* **1992**, 625–626. (e) Merkler, D. J.; Kulathila, R.; Tamburini, P.; Young, S. D. *Arch. Biochem. Biophys.* **1992**, *294*, 594–602. (f) Zabriske, T. M.; Cheng, H.; Vederas, J. C. *J. Am. Chem. Soc.* **1992**, *114*, 2270–2272. (g) Katapodis, A. G.; May, S. W. *Biochemistry* **1990**, *29*, 4541–4548. (h) Freeman, J. C.; Villafranca, J. J.; Merkler, D. J. *J. Am. Chem. Soc.* **1993**, 115, 4923–4924.
(38) (a) Casella, L.; Gullotti, M.; Bartosek, M.; Pallanza, G.; Laurenti, E. *J. Chem. Soc., Chem. Commun.* **1991**, 1235–1236. (b) Casella, L.; Gullotti, M. In *Bioinorganic Chemistry of Copper*; Karlin, K. D., Tyeklar, Z., Eds.; Chapman & Hall: New York, 1993; pp 292–303.

Table 3. Metrical Parameters Used in the Simulation of the EXAFS Spectra of the CO Complex of Selectively Demetalated Dopamine-β-hydroxylase Sample H, Compared with Those of the CO Derivative of the Fully Demetalated Enzyme

fully metalated CO			selectively demetalated CO		
shell	distance (Å)	Debye–Waller (Å²)	shell	distance (Å)	Debye–Waller (Å²)
2.5 N (His)[a]	1.95	0.011	2 N (His)[a]	1.94	0.009
0.5 S	2.28	0.008	0.85 S	2.26	0.011
0.5 CO[b,c]	1.78	0.006	0.85 CO[b,c]	1.76	0.010

[a] The histidine rings were simulated as geometrically rigid units, using the multiple scattering protocols of the program EXCURV, as described under methods. Thus, the fits all contain outer shell C and N atoms at the appropriate positions for an imidazole ligand with Cu–N bond lengths as given in the table. Full details of the imidazole ring geometrical parameters are given in the supplementary material. [b] The carbonyl group was simulated as a linear unit using the multiple scattering methods of the program EXCURV. The O atom associated with the CO group refined to 2.99 ± 0.02 Å in both fully metalated and demetalated derivatives, giving a C–O distance of 1.23 Å in both cases. [c] The shell occupancy (coordination number) for the CO group was set at the analytically determined value. This was 0.51 for fully metalated and 0.85 for selectively demetalated sample H.

at 1945 cm^{-1} (nonlinear CO),[25,39] whereas ν_{CO} of the nonlinear P450-CO is at 1940–1942 cm^{-1}.[40] This comparison indicates that thiolate is expected to decrease ν_{CO} relative to imidazole but is complicated by the nonlinearity of the Fe–C–O unit and the dependence of ν_{CO} on the Fe–C–O angle in these systems. A similar comparison of the $Fe(T_{piv}PP)XCO$ system (picket fence porphyrin) by Collman and Sorrell[41] gave ν_{CO} of 1964 cm^{-1} for X = 1-MeIm, 1945 cm^{-1} for X = CH_3S^-, and 1970 cm^{-1} for X = n-C_3H_7SH. For the five-coordinate Fe(TPP)CO system Wayland and co-workers reported ν_{CO} = 1973 cm^{-1}.[42] These trends show that as expected ν_{CO} decreases with increasing basicity of the axial ligand to the heme and that thiolate is a better donor than imidazole, leading to lower carbonyl stretching frequencies. On the other hand, protonation of the thiolate to a thiol appears to eliminate the donor effect of the axial thiolate, giving ν_{CO} comparable with that of the five-coordinate Fe(TPP)CO complex. Thus whereas thiolate can in all likelihood be eliminated as the origin of the S in Cu_B of DBH (since ν_{CO} is greater than the value expected for a trisimidazole system), a protonated thiol cannot be excluded on the basis of IR data alone.

Thiol coordination is attractive mechanistically since it would establish a mechanistic homology with the cytochrome P450 family of enzymes which all contain an axial thiolate ligand coordinated to the heme. This thiolate is believed to assist the catalysis in P450 by virtue of its strong electron donation which helps to stabilize the ferryl intermediate formed by heterolysis of the O–O bond.[43] Additionally, thiolate coordination renders the Fe^{2+} form of the enzyme accessible for O_2-binding, unlike catalase and certain peroxidases in which the harder phenolate and imidazole ligation stabilizes the ferric states relative to the ferrous.[43] This chemistry may also be relevant to the DBH mechanism. Klinman has recently shown that the observed ^{18}O isotope effects in a series of fast and slow substrates can only be explained if it is assumed that O–O bond fission precedes H-atom abstraction from the phenylethylamine substrates.[33] In her new mechanism, she suggests that the initial $Cu^{II}OOH$ intermediate abstracts an H atom from an active site tyrosine residue and thereby induces fission of the O–O bond to produce water and

(39) (a) Alben, J. O.; Caughey, W. S. *Biochemistry* **1968**, *7*, 175–183. (b) Tsubaki, M.; Srivastava, R. B.; Yu, N. T. *Biochemistry* **1982**, *21*, 1132–1139.
(40) O'Keefe, D. H.; Ebel, R. E.; Peterson, J. A.; Maxwell, J. C.; Caughey, W. S. *Biochemistry* **1978**, *17*, 5845–5852.
(41) Collman, J. P.; Sorrell, T. N. *J. Am. Chem. Soc.* **1975**, *97*, 4133–4134.
(42) Wayland, B. B.; Mehne, B. F.; Swartz, J. *J. Am. Chem. Soc.* **1978**, *100*, 2379–2383.
(43) Dawson, J. H.; *Science* **1988**, *240*, 433–439.

a copper(II)–oxygen radical species Cu–O•. This radical contains one formal oxidizing equivalent, and resonance forms of the $Cu^{II}O^{•}$ species can be written in which the oxidizing equivalent is delocalized from oxygen to copper producing a formal copper(III)–oxo species. Thiol coordination could provide a mechanism for stabilizing this species via electron donation from S to Cu(III) and partial proton transfer from sulfur to O to form a thiolate–Cu^{III}–OH or S•–Cu^{II}–OH intermediate. S-Donation is known to stabilize Cu(III) complexes such as *N,N'*-dialkyldithiocarbamates,[44] and hence thiol coordination at Cu_B in dopamine-β-hydroxylase could fulfill a similar role to thiolate in P450 chemistry.

Despite this rather attractive speculation that a thiol ligand might provide the "push" analogous to cysteinate in P450 monooxygenations, there are serious objections to thiol coordination on chemical grounds. To our knowledge, no thiol-coordinated model complexes of either Cu(I) or Cu(II) exist. Notwithstanding, Cu(I) is a weak Lewis acid, and might conceivably allow coordination of a protonated thiol in a protein environment; however, oxidation to the Cu(II) form would induce deprotonation and coordination of thiolate, due to the increased Lewis acidity of the Cu(II), and no evidence of intense thiolate-to-copper(II) charge transfer has ever been observed in oxidized DBH, effectively excluding thiolate from the vicinity of the Cu(II) coordination sphere.

Clearly further work is necessary before any firm conclusions can be reached concerning the mechanistic role of the S atom at Cu_B. At this time we should not exclude an alternative explanation that the ascorbate-reduced enzyme is a resting form and that the S-ligand is not present in the catalytic form. Such might be the case if the active dioxygen-binding form were a highly distorted two-coordinate species with a strong driving force for coordinating a third ligand which, in the absence of dioxygen, could be methionine. This seems most unlikely on several grounds. Firstly CO and O_2 compete for the same site,[9] and the Cu_B–CO derivative is clearly four-coordinate and contains the S ligand; secondly Klinman has demonstrated the catalytic competence of the ascorbate-reduced enzyme in single turnover experiments;[14] thirdly substrate binding induces little change in Cu(I) structure and does not displace the S as indicated by EXAFS,[12] and hence cannot be a trigger for conversion from resting (sulfur-bound) to active (sulfur-free) states. All these arguments lead to the conclusion that methionine coordination is most consistent with all the data. Further work is underway to establish the relevance of the S coordination in the catalysis.

(44) Cotton, F. A.; Wilkinson, G. *Advanced Inorganic Chemistry*, 5th ed.; John Wiley: New York, 1988; p 774.

Acknowledgment. We acknowledge the use of synchrotron radiation facilities and the help given us by personnel at NSLS, particularly Dr Sayed Khalid. This work was supported by grants from the National Institutes of Health to N.J.B., R01 NS-27583, and to the National Biostructures PRT and the NSLS Brookhaven National Lab, RR01 633–10.

Supplementary Material Available: Full parameter sets used in the simulation of the data in Figures 3a–c and 4a,b, as output by the program EXCURV88, together with full documentation of the definition of these parameters; contour maps of the three-dimensional surface describing the variation of least-squares fit index as a function of CO and S coordination number and Debye–Waller factors for half-apo carbonyl DBH sample H (9 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.