Notes

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Copper X-ray Absorption Spectroscopic Studies of the Bovine Plasma Amine Oxidase–Sulfide Complex

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Bovine plasma amine oxidase is a copper-containing enzyme (2 Cu atoms/protein molecule)[3] which also contains an organic cofactor that displays the reactivity of a carbonyl group but that has proved difficult to definitively identify. There is now considerable evidence that pyrroloquinoline quinone (PQQ), or a closely similar derivative, is the organic cofactor in this class of amine oxidases.[4-9] Both the copper and the organic cofactor are required for activity, and recent work is consistent with a 1:1:1 copper:cofactor:subunit stoichiometry.[10] Substrate amines react with the organic cofactor and are oxidized to aldehydes, thereby reducing the enzyme by two electrons; copper is required for the subsequent oxidation of the reduced enzyme by O_2, producing H_2O_2 and (generally) releasing NH_3.[3] Elucidating the structural and functional relationships between the cofactors is therefore critical for any fundamental understanding of amine oxidase catalysis.

NMR[11] and fluorescence energy-transfer data[12] indicate that the copper ions and the reactive quinone carbonyl group(s) are separated by several angstroms. Nevertheless, numerous experiments point to significant interactions between copper and the cofactor.[13] In particular, the irreversible inactivation of bovine plasma amine oxidase by sulfide[14] and the effects of cyanide on the substrate-reduced amine oxidases[15] indicate that the electron transfer between the copper ions and PQQ is possible. In the former case, HS^- was proposed to initially bind to the enzyme, forming a Cu^{II}–SH^- complex, which then underwent further reactions to eventually give Cu(I) and the reduced organic cofactor as products. Given the key role suggested for the Cu^{II}–SH^- complex in the inactivation reaction, we decided to characterize the structure more completely by using X-ray absorption spectroscopy (XAS). A great deal of work has established that both the copper K-edge and extended X-ray absorption fine structure (EXAFS) are quite sensitive to (and hence diagnostic for) sulfur coordination.[16,17]

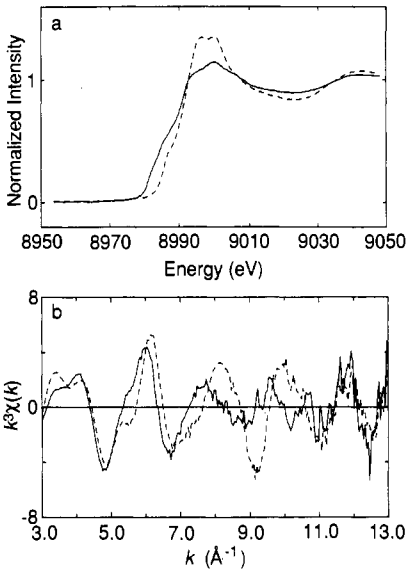

Figure 1. Copper K absorption edge (a) and EXAFS (b) spectra for resting (oxidized) bovine plasma amine oxidase (—) and its complex with sulfide (---).

Experimental Section

Bovine plasma amine oxidase was purified by modifications of published procedures.[18] Samples used for XAS were highly homogeneous as judged by SDS and gradient polyacrylamide gel electrophoresis; specific activities exceeded 0.25 unit mg^{-1}, assayed with benzylamine. (One enzyme unit catalyzes the oxidation of 2 μmol min^{-1} of benzylamine to

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Table I. Curve-Fitting Results for the First Coordination Spheres of Resting and Sulfide-Treated Bovine Plasma Amine Oxidase[a]

		Cu–(N,O)			Cu–S			
sample	fit	N	R, Å	$\Delta\sigma^2$, Å^2	N	R, Å	$\Delta\sigma^2$, Å^2	f'[b]
resting	1	(4)[c]	1.98	+0.0019				0.041
	2	(3)	1.98	+0.0002	(1)	2.30	+0.0245	0.048
resting + sulfide	3	(3)	2.02	+0.0026	(1)	2.32	+0.0037	0.038
	4	(2)	2.02	−0.0000	(1)	2.30	+0.0025	0.034
	5	(4)	2.04	+0.0052				0.094

[a] N is the number of scatterers per copper; R is the copper–scatterer distance; $\Delta\sigma^2$ is the relative mean square deviation in R, $\Delta\sigma^2 = \sigma^2$(sample) − σ^2(reference), where the reference is $[Cu(im)_4]^{2+}$ at 4 K for Cu–(N,O) and $[Cu(mnt)_2]^{2-}$ at 4 K for Cu–S (im = imidazole; mnt = maleonitriledithiolate). All fits were over the range k = 4.0–12.0 Å^{-1}. [b] f' is a goodness-of-fit statistic normalized to the overall magnitude of the $k^3[\chi(k)]$ data:

$$f' = \frac{\{\sum[k^3(\chi_{obsd}(i) - \chi_{calcd}(i))]^2/N\}^{1/2}}{(k^3\chi)_{max} - (k^3\chi)_{min}}$$

[c] Numbers in parentheses were not varied during optimization.

benzaldehyde.) Amine oxidase samples for XAS were exhaustively dialyzed against metal-free 0.1 M KPO_4 buffer (pH 7.0) containing 2 mM EDTA followed by dialysis against the metal-free buffer alone. Solutions were concentrated to approximately 1 mM protein in a vertical, semipermeable membrane device from Biomolecular Dynamics. Glycerol was then added (in order to facilitate glass formation upon freezing), giving a final concentration of 0.75 mM protein (1.5 mM Cu). Trace metal ions were removed from the buffer by passage over a Chelex column; the effectiveness of this technique was confirmed by atomic absorption spectroscopy. Sodium sulfide solutions were prepared immediately before use. The sulfide complex of resting bovine plasma amine oxidase was prepared by adding a concentrated sulfide solution, sufficient to give a sulfide concentration equal to 20[Cu], to the enzyme in the Lucite XAS cuvette and rapidly (<30 s) freezing the sample in liquid nitrogen. Significant reduction to Cu(I) is unlikely under these conditions as judged by EPR, activity, and optical measurements.[14]

Copper XAS data were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) on the wiggler beam line IV-1 with the SPEAR ring operating under dedicated conditions (3.0 GeV, ~70 mA). The X-ray beam was monochromated by using Si[220] crystals, and energy calibration was performed using the internal calibration technique[16] 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 10 K as frozen solutions in a custom-made Oxford Instruments cryostat (now Model CF1208). Each EXAFS spectrum consists of 20 signal-averaged 23-min scans covering the 8650–9700-eV range, whereas each high-resolution edge spectrum required signal averaging of only two 14-min scans. Data reduction and analysis were accomplished by our standard methods,[17] internally calibrating to the first inflection point of Cu foil as 8980.3 eV and using E_0 = 9000 eV.

Results and Discussion

The effects of sulfide on the Cu(II) K-edge of bovine plasma amine oxidase are shown in Figure 1a. As noted previously,[19] the Cu(II) edge of the oxidized enzyme is very similar to that of $[Cu(im)_4]^{2+}$ (im = imidazole). Both the shift to lower energy and the intensity decrease of the maximum edge jump are consistent with at least one sulfur ligand in the first coordination shell of Cu(II) in the amine oxidase–sulfide complex.[17] Modeling studies indicate that the higher covalency and charge delocalization associated with Cu^{II}–S bonding can adequately account for the shape and lower energy of the Cu(II) edge in complexes containing sulfur-donor ligands.[17] It is clear that the Cu(II) EXAFS is also altered significantly by sulfide (Figure 1b). EXAFS curve-fitting results for the first coordination sphere are shown in Figure 2, and the resulting structural details are given in Table I.[20] The fit to the filtered data for the sulfide complex obtained by assuming one S-containing and three N,O-containing ligands (fit 2 in Table I) is displayed in Figure 2d. These results conclusively demonstrate that sulfide (probably as HS^-) binds directly to Cu(II) and are

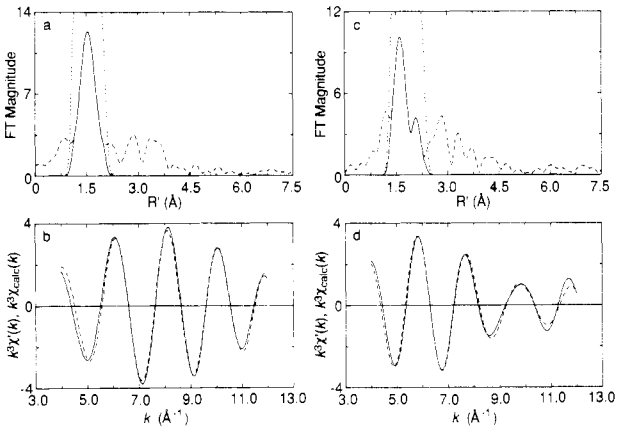

Figure 2. Fourier transforms (k = 3.0–13.0 Å^{-1}, k^3 weighted) of the copper EXAFS spectra of resting bovine plasma amine oxidase (a) and its sulfide complex (c). In each, the dotted lines represent the filter windows used to extract the first coordination sphere EXAFS shown as solid lines in (b) and (d). The dashed lines in (b) and (d) represent the best curve-fitting simulations from Table I (fits 1 and 3, respectively).

entirely consistent with previous spectroscopic data.[14] Although this is the first determination of a copper–sulfur bond length (2.31 Å) in a Cu(II)–sulfide complex, the observed bond length is within the range (2.30–2.34 Å) generally found for equatorial thioether ligands in tetragonal Cu(II) complexes.[21] A Cu^{II}–S(cys) distance of 2.28 Å was found for the Cu_A site in bovine cytochrome c oxidase.[17] The fits (Table I) also indicate that one or perhaps two N,O-ligands are lost concomitantly with sulfide binding. Since the most strongly bound ligands in a tetragonal Cu(II) complex are equatorial and therefore normally dominate the copper EXAFS, the coordination numbers in the EXAFS fits primarily reflect the composition of the equatorial plane.[16] Hence, sulfide binding to bovine plasma amine oxidase may be accurately described as an equatorial ligand-substitution reaction. The data reported here support the earlier suggestion[14] that coordination is the initial step in the inactivation of bovine plasma amine oxidase by sulfide. Copper-containing amine oxidases display facile ligand-substitution chemistry[22,23] with equatorially coordinated H_2O generally serving as the leaving group.[22-24] Exogenous ligand

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binding to these amine oxidases invariably produces inhibition.[23,25] It is therefore possible that inactivation is associated with irreversible binding.

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Oligomeric Iminolithium and Amidolithium Compounds: Comments on Previously Reported Theoretical Calculations and Experimentally Determined Structures

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In a recent article,[1] Raghavachari, Sapse, and Jain (henceforth RSJ) described ab initio calculations for prediction of the structures and energies of the hexamers of LiF, LiOH, and $LiNH_2$, and concluded that in all three cases, a D_{3d} clustered structure based on a distorted Li_6 octahedron is significantly more stable than a D_{6h} planar hexagonal ring structure. Contrary to statements by RSJ, our own experimental results,[2] by X-ray crystallography, for the structures of some hexameric iminolithium compounds $[LiN{=}CRR']_6$ (R = Ph, R′ = *t*-Bu; R = Ph, R′ = NMe_2) do *not* provide direct support for their predictions. The RSJ discussion overlooks features of imides $[LiN{=}CRR']_n$ and amides $[LiNRR']_n$ that are quite different and can be demonstrated from experimentally observed structures of both classes of compounds.

First, the interpretation of our structural results on the iminolithium compounds $[LiN{=}CRR']_6$ by RSJ is incorrect and is at variance with our own already published interpretations. The RSJ calculations for $[LiNH_2]_6$ indicate two "short" Li–N bonds at 1.99 Å and one "long" bond at 2.06 Å for each triply bridging nitrogen atom.[1] The iminolithiums display average lengths of 1.98, 2.01, and 2.05 Å for the Li–N bonds.[2] The "slight asymmetry seen between the two short bonds" is attributed by RSJ to crystal packing effects. This difference is, however, genuine: the short–medium–long pattern of three Li–N bonds is observed for *every* bridging nitrogen in *both* of the molecular structures reported by us[2] (one of them with two crystallographically independent molecules in different environments) and in a previously determined iminolithium hexamer;[3] i.e. this pattern is observed, *without exception*, for no fewer than 24 NLi_3 units. The pattern is too regular to be caused by low-symmetry crystal packing, which in any case is different for each of the structures.

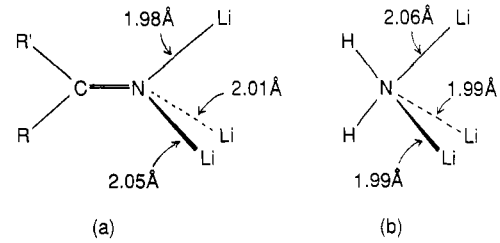

Figure 1. Orientations of (a) the imido groups N=CRR′ and (b) the amide groups NH_2 with respect to the bridged triangles of metal atoms in (a) experimentally observed $[LiN{=}CRR']_6$ and (b) calculated $[LiNH_2]_6$.

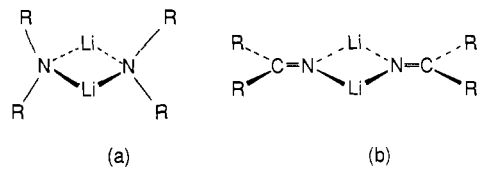

Figure 2. Different positions of substituents R in cyclic lithium amides $[LiNR_2]_n$ (a) and lithium imides $[LiN{=}CR_2]_n$ (b) as typified by dimers, $n = 2$.

What the short–medium–long pattern does clearly reflect, *in every case*, is the orientation of the imino unit N=CRR′ with respect to the Li_3 triangle it bridges (Figure 1a).[4] Invariably, the short Li–N contact lies in, or nearest to, the N=CRR′ skeletal plane (the plane in which an anion $[N{=}CRR']^-$, with an sp^2-hybridized nitrogen atom, would have lone-pair electron density), while the medium and long Li–N bonding contacts involve pairs of metal atoms that straddle this plane. Their lengths thus reflect differences in electron density between the metal and nitrogen atoms. The Li^+ metal ions are closest to N in those directions where the lone-pair electron density is greatest. Although the bonding in these systems is highly polar, indeed largely ionic, one can effectively rationalize the *differences* in their metal–nitrogen distances in terms of two- and three-center bonding interactions—a short, two-center LiN bond to the metal atom in the N=CRR′ skeletal plane, and an unsymmetrical three-center Li_2N bond to the pair of metal atoms straddling that plane. The short–medium–long pattern of Li–N distances experimentally observed for the iminolithium hexamers is thus intimately bound up with the ligand skeletal plane orientation and is an intrinsic geometrical feature of the molecule and not a consequence of its crystalline environment. Although we have only recently discussed this in detail,[4] the main points of our argument were made quite clear in the preliminary report[2] misinterpreted by RSJ.[1] In the RSJ calculations for $[LiNH_2]_6$, the amido groups NH_2 (which are not isolobal with imino groups N=CRR′) have an orientation that is consistent with two strong short bonding NLi contacts to two of the three neighboring atoms, the third lying in a direction apparently more suited to a longer three-center NHLi interaction than to involvement of nitrogen lone-pair electron density (Figure 1b). The equivalence of the two shorter Li–N distances is a reflection of the orientation of the NH_2 plane relative to the Li_3 triangle. This is one fundamental difference between RSJ's $[LiNH_2]_6$ geometry and the experimentally observed geometry for the $[LiN{=}CRR']_6$ structures.

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