activation enthalpy is cancelled by an important relative loss of entropy (10 eu) compared with toluene, with the result that the two solvents have a similar efficiency.

**Arguments in Favor of a Symmetrical Triple Exciplex.** 1. From the above spectroscopic studies it appears that only two main sets of conformers participate in the photophysics of AN2. Of note is the strong tendency of the molecule to adopt the highest symmetric geometry throughout a wide temperature range (−80 + 90 °C) in the ground state as demonstrated by NMR ($C_{2v}$ symmetry). This is why the two sets have been denoted "in–in" and "out–out" and why the "in–out" geometry has been discarded.

2. A symmetrical exciplex can be built up from the "in–in" b* conformer with the minimum of atomic movement and a weak activation energy (2.2 kcal/mol); thus, the lone pairs of the two nitrogen atoms can simultaneously experience the best overlap with the $\pi$ electron cloud at the meso positions, i.e., those of the highest electronic density. Inspection of molecular models suggests that an "in–out" conformation for AN2 conducive to a mononitrogen exciplex would be strained.

3. Single crystals of AN2 (grown in toluene solutions) emit only the exciplex fluorescence[7c,10] with a decay of 18 ns at 20 °C, which is of the same order of magnitude as one of the components of the exciplex emission in toluene and dichloromethane; the spectrum is similar in shape and in maximum wavelength ($\lambda_{max} \approx 520$ nm) to those obtained from toluene (490 nm) and dichloromethane (520 nm). Moreover, owing to the large distance between the parallel anthracenic rings (≈10–11 Å) in the crystal, intermolecular excimer formation can be ruled out. In the solid state, the two nitrogen lone pairs are symmetrically oriented *inside* the cavity ("in–in").[10] That the conformation "b" which, in fluid solution, leads to the exciplex and which is dominant at low temperature ([b]/[a] ≈ 15 at −90 °C) has the same geometry as that found in crystals is beyond doubt. In contrast, the "a" species, which absorbs light like the reference compound (R), should exhibit "out–out" orientation of its nitrogen lone pairs presumably orthogonal to the $\pi$ aromatic electrons, in agreement with the absence of interaction between the chromophores. This conclusion is reinforced by inspection of molecular models which indicate that "b" and "a" conformers, although both strained, could be in interconversion.

## Conclusion

We have described an excited-state complex between two aliphatic amines and one aromatic hydrocarbon, which is postulated to be the first symmetrical nonlinear triple exciplex.

This new exciplex has a relatively high fluorescence quantum yield compared to other triple exciplexes. A detailed kinetic and thermodynamic analysis allowed a description of the molecular dynamics of the system.

**Acknowledgment.** Many thanks are due to B. Barbe for measuring the NMR spectra. We are very grateful to Drs. B. Clin and C. Naulin for assistance in the NMR data analysis. The authors are indebted to Dr. C. Rullière for recording the picosecond time-resolved spectra. We are indebted to the referees for their judicious and stimulating remarks.

**Supplementary Material Available:** Tables of decay parameters of AN2 as a function of temperature (2 pages). Ordering information is given on any current masthead page.

# EXAFS Studies on Pig Plasma Amine Oxidase. A Detailed Structural Analysis Using the Curved Wave Multiple Scattering Approach

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**Abstract:** EXAFS studies have been carried out on pig plasma amine oxidase containing one copper and two copper atoms. The data for the two forms are essentially identical and are consistent with the presence of four low-Z scatterers and one heavier scatterer in the primary ligand shell. There is evidence that the heavy scatterer is a sulfur atom coordinated at 2.38 Å. The four low-Z scatterers distribute themselves about the copper atom at two discrete distances, 2.00 and 1.90 Å. The 2.00-Å distance is accounted for by two nitrogens and the outer shell analysis is consistent with this as imidazole coordination. The two low-Z scatterers at 1.90 Å are consistent with Cu–O coordination, probably arising from phenolate and/or $OH^-$. The analysis provides no direct evidence for or against the presence of PQQ coordinated to copper.

Amine oxidases catalyze the oxidative deamination of amines to the corresponding aldehyde, hydrogen peroxide and ammonia. There are two categories of amine oxidase: those containing copper (E.C.1.4.3.6) and those containing flavin (E.C.1.4.3.4). Both categories of amine oxidase have important roles in the catabolism of biogenic amines, including histamine, serotonin, and catecholamines, while the copper-containing enzymes are also involved in cross-linking reactions in connective tissue.[1]

The copper-containing amine oxidase from pig plasma (PPAO) is one of the better characterized in this class of enzyme. The homogeneously pure enzyme has a molecular weight of 190 000 composed of two subunits with equal molecular weight.[2,3] It is known that the enzyme contains a novel cofactor pyrrolo quinoline quinone (PQQ) in addition to copper.[4] The structure and function

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of the two copper sites in the enzyme, which have been shown to be chemically distinct by ESR spectroscopy,[3,5] are not understood in detail. It is known from ESR studies that the $Cu^{2+}$ is tetragonally coordinated and that there is no "through-space" interaction between the two coppers. The results of water proton relaxation[3] and ENDOR[6] studies indicate that there are two water molecules (one equatorial, the other axial) coordinated to the copper. With respect to the function of the copper sites, the evidence suggests that copper is essential for catalytic activity, probably in reactions involving reoxidation of substrate-reduced enzyme by oxygen. It has been observed[5] that copper can be removed completely from PPAO and 50% activity restored by addition of one $Cu^{2+}$ per molecule of enzyme. Thus we can now begin to compare the structural characteristics of native (2-$Cu^{2+}$) and 1-$Cu^{2+}$ forms of the enzyme.

X-ray absorption spectroscopy (EXAFS and XANES) has been used extensively in defining the structure of metal sites in enzymes.[7] A particular advantage of EXAFS is the ability to determine bond lengths in either crystalline or noncrystalline samples of metalloproteins to a greater precision than that obtained in crystallography. We have successfully used this technique to investigate other type 2 copper proteins: namely Cu–Zn superoxide dismutase[8] and dopamine-$\beta$-hydroxylase.[9] Scott and Dooley[10] have compared the EXAFS data of amine oxidase with $Cu(Imid)_4(NO_3)_2$ to show that the Cu atom is coordinated to low-Z ligands in a similar manner. Recent advances in the techniques of EXAFS analysis[11] has meant that important multiple scattering interactions can now be treated routinely. We have used curved wave multiple scattering formalism to simulate the EXAFS of a number of copper–imidazole[8c,12] and copper–pyridine[13] complexes such that all the ring atoms are located at their correct distances. The overall shape of the XANES region of the X-ray absorption spectrum is well reproduced too when multiple scattering contributions up to third order are included in the calculations. This approach has been particularly useful in defining the Cu site in superoxide dismutase as a function of anion binding.[8c] In the present paper we apply the multiple scattering treatment to the EXAFS of pig plasma amine oxidase which gives a more detailed model for the Cu site in this enzyme.

## Materials and Methods

Pig plasma amine oxidase (PPAO) was purified as described by Rius et al.[14] and extraneous copper removed by passage through a column of chelating resin (Sigma, UK) equilibrated in 20 mM Pipes buffer at pH 7.0. The "1-Cu" form of PPAO was prepared as described by Collison et al.[5] through addition of stoichiometric amounts of $Cu(NO_3)_2$ to a concentrated solution of the apo-enzyme in 20 mM Pipes buffer at pH 7.0 and incubation of the reconstituted sample at 20 °C for 8 days with occasional stirring. The reconstituted "1-Cu" enzyme had 50% of the activity of native enzyme; full activity could be restored to the "1-Cu" enzyme by the addition of a second stoichiometric amount of $Cu(NO_3)_2$. Both native and "1-Cu" enzyme samples were concentrated by pressure dialysis to a concentration of 200 mg per mL and then loaded into the EXAFS cell.

EXAFS measurements were recorded in the fluorescence mode as fluorescence excitation spectra[15] on the wiggler station 9.2 at the SRS, Daresbury Laboratory, operating at an energy of 2 GeV and a maximum beam current of 300 mA. A Si220 double-crystal order-sorting monochromator was used to provide monochromatic beam. The enzyme samples were maintained at 77 K during the measurements with an

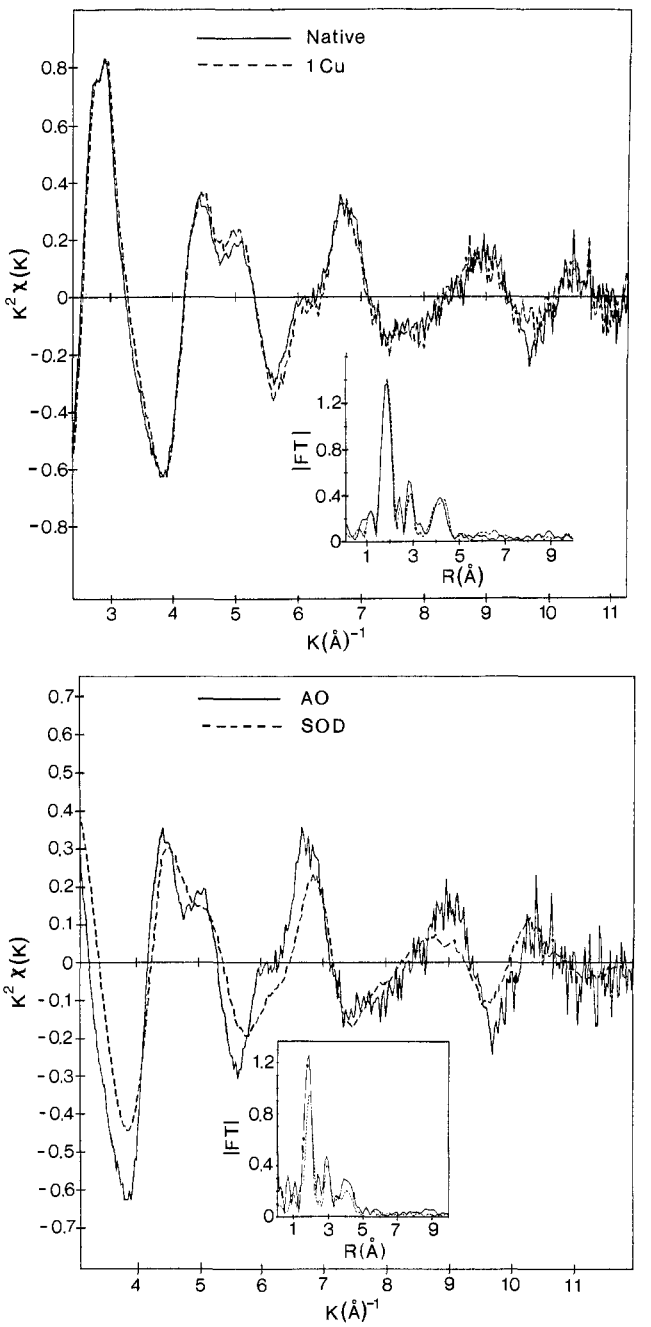


Figure 1. $K^2$-weighted experimental Cu K-edge EXAFS and Fourier transforms for (a, top) native amine oxidase (solid line) and 1-Cu amine oxidase (dotted line) and (b, bottom) native amine oxidase (solid line) and native superoxide dismutase (dotted line).

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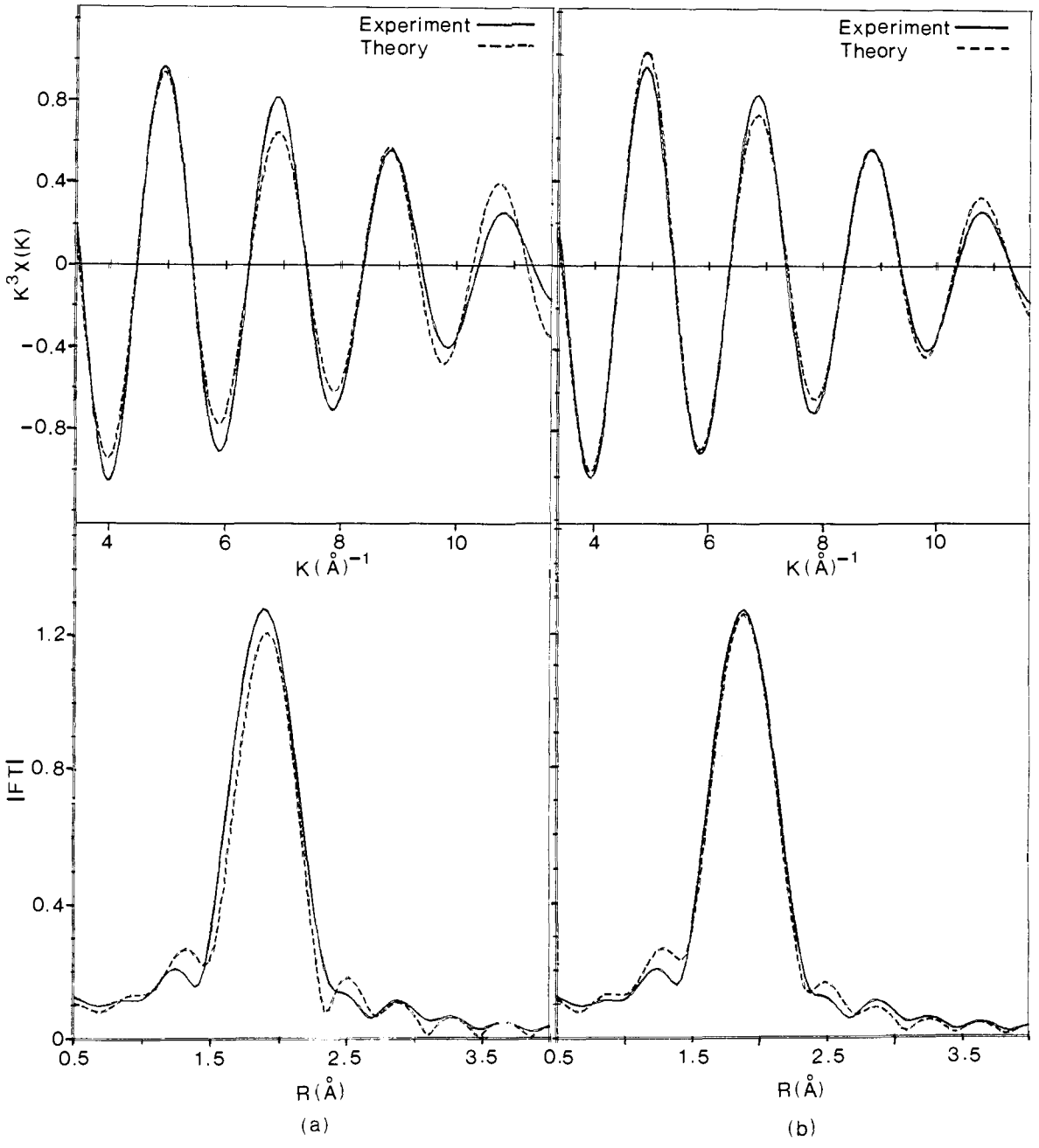


**Figure 2.** $K^3$-weighted simulation of the Fourier filtered first shell of native amine oxidase showing (a) the fit obtained for 4 nitrogens at 1.94 Å and (b) the fit obtained for 2 oxygens at 1.90 Å and 2 nitrogens at 2.00 Å.

Oxford Instruments continuous flow helium cryostat. For both native and 1-Cu samples, a total of 12 scans were recorded. The averaged spectra were normalized and background subtracted as previously described.[8] The EXAFS thus obtained was converted to $k$-space and weighted by $k^3$ to compensate for the diminishing amplitude at high $k$. Data analysis was carried out with the curved wave method for calculating EXAFS with phase shifts derived from ab initio calculations as previously described.[16] EXAFS simulations were performed with the program EXCURVE[11a] as described in detail elsewhere.[8,17] The experimental spectrum is shifted by using a parameter $\Delta E_0$ to match with the theoretical simulation, as described previously.[8,16b] This has the effect of putting the top of the absorption edge at $K \approx 3$ Å$^{-1}$. Fourier transforms are corrected for the nearest-neighbor shell phase shift. This results in placing the FT peaks at approximately the correct distance. Fourier transform is used as a guide for assessing the status of a particular model and the quality of simulation for the EXAFS data.[16b]

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## Results and Analysis

Figure 1 shows the EXAFS spectra and Fourier transforms for both enzyme samples; also shown in Figure 1 is a comparison with superoxide dismutase (SOD). The two samples of amine oxidase give essentially identical spectra, thus suggesting that structural differences between the Cu sites are minimal. The spectrum of amine oxidase shows similarity to that of the superoxide dismutase; however, the EXAFS data of the two enzymes are distinct. For example, the phase difference between the two spectra suggests an overall shorter distance for the primary shell in the amine oxidase. This is evident in the Fourier transforms, which also show a significant contribution at about 2.4 Å for amine oxidase. These qualitative observations are borne out by detailed analysis presented below.

The starting point for our analysis was the EXAFS result for oxidized SOD[8] which consists of a Cu–N(His) at 2.0 Å and a Cu–O(water) at 2.24 Å. Refinement of the inner-shell parameters on both Fourier filtered and raw data suggests the presence of a minimum of four low-Z scatterers in the first shell with an average distance of 1.94 Å and a heavier atom at about 2.4 Å.

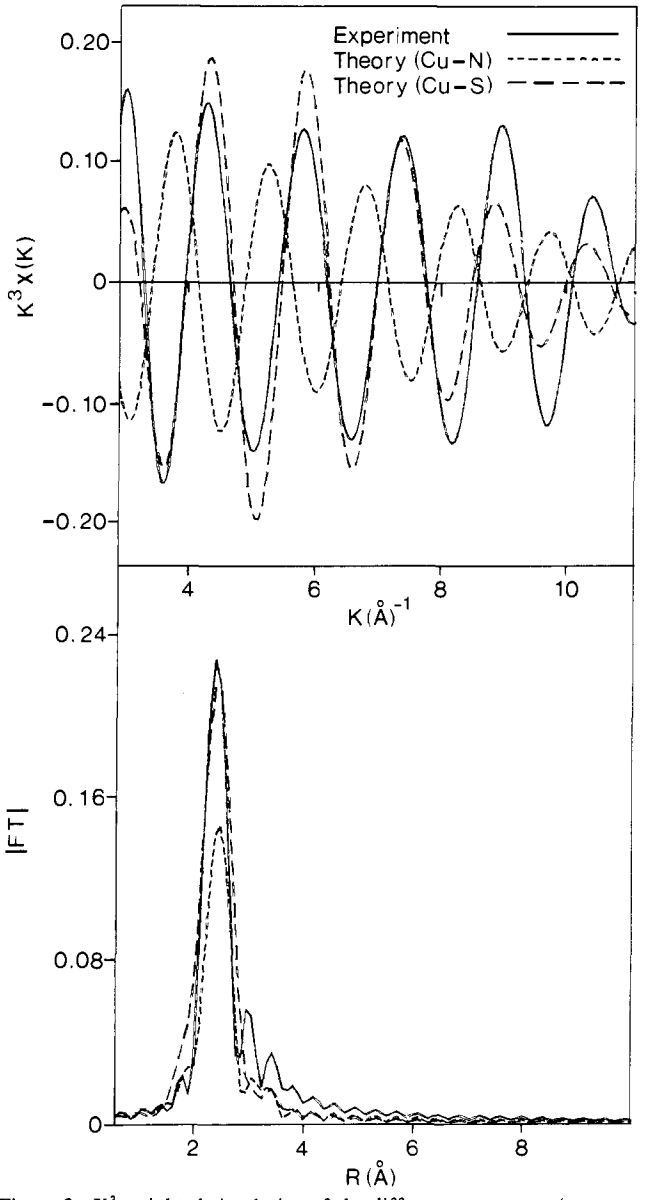


Figure 3. $K^3$-weighted simulation of the difference spectrum (see text for details) for (a, top) Cu–S and (b, bottom) Cu–N backscattering at 2.4 Å.

Simulations of the Fourier filtered first shell were carried out for four low-Z scatterers (nitrogens) at the same distance, which refined to 1.94 Å with a fit index of 0.82 (Figure 2a). The simulation is slightly improved by allowing the positions of the four atoms to refine separately. The refinement gave two discrete distances with two nitrogens at 1.91 Å and two at 1.97 Å, and a fit index of 0.70. A major improvement was obtained when the shorter contact was assumed to be due to oxygen. In this case the fit index reduced to 0.20. This large improvement in fit index results primarily from the much improved nature of the phase particularly in the $k$ range 9–11 Å$^{-1}$ when oxygen is used. We note that such a distinction between oxygen and nitrogen is not always possible. The best fit thus obtained is with two nitrogens at 2.00 Å and two oxygens at 1.90 Å (Figure 2b). A simulation of the raw EXAFS data taken over the whole range with this model also gives a superior fit index (12.9) compared to when all four ligands are assumed to be N (14.9). The value of the Cu–N distance is in the range expected of Cu–N(histidine) coordination,[18] whereas the shorter distance of 1.90 Å is compatible with a phenolate or $OH^-$ linkage.[19]

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Table I. Parameters Used To Simulate the Theoretical EXAFS Fits Shown in Figures 4 and 5 (Estimated Errors are ±0.02 Å for Inner-Shell and ±0.04 Å for Outer-Shell Distances)

| shell | $R$/Å | $2\sigma^2$/Å$^2$ | BA[b] |
|---|---|---|---|
| 2 O | 1.90 | 0.003 | |
| 1 S | 2.38 | 0.016 | |
| 2 N(1mid)[c] | 2.00 | 0.003 | |
| 4 C(1mid)[c] | 2.91 | 0.006 | 120 |
| 2 N(1mid)[c] | 4.14 | 0.008 | 163 |
| 2 C(1mid)[c] | 4.14 | 0.008 | 163 |
| 1 C[a] | 2.97 | 0.010 | |
| 1 C[a] | 3.16 | 0.010 | |
| 3 C[a] | 4.30 | 0.010 | |

[a] These "extra" atoms are included in the fits shown in Figures 4b and 5. [b] BA is the angle used to define multiple scattering interactions for the imidazole rings; e.g., multiple scattering for the pathway Cu–N1–C2/C5 is calculated for an angle of 120°. [c] These atoms belong together to two imidazole groups. Corresponding distances for Cu(1mid)$_4$(NO$_3$)$_2$ are 2.00, 2.94, 4.14, and 4.18 Å.

The Fourier transform shown in Figure 1a indicates the presence of at least one scattering atom at about 2.4 Å. We have tried fitting both the Fourier filtered and the raw EXAFS data with low-Z scatterers at this distance without success. This shell is well reproduced, however, by a sulfur atom. In order to demonstrate that this is due to a sulfur atom, a difference method was employed which has proven useful in the case of azurin for identifying a S(Met).[20] In view of the overlapping nature of the Fourier transform peaks, both the primary shell peak and the peak at about 2.4 Å were backtransformed. EXAFS of the primary shell was simulated by two O at 1.90 Å and two N at 2.00 Å which, when subtracted from the Fourier filtered data, give the contribution due to the 2.4-Å shell. This is shown in Figure 3, where attempts to simulate this contribution with a nitrogen atom and a sulfur atom (refining to 2.38 Å) are shown. Due to the phase difference between the experimental wave and the simulated Cu–N wave, this contribution can be unambiguously attributed to a scatterer such as S or Cl; the distance of 2.38 Å indicates a thioether rather than a thiolate if sulfur is the coordinating atom.[21] A simulation of the complete raw EXAFS data is also consistent with this conclusion, which showed an improvement in fit index value of 25% when S was included in the model. A simulation of the Fourier-smoothed data gave an improvement of approximately 40% in the fit index when S was included in the model.

We have examined the possibility of imidazole coordination using the multiple scattering method previously established for other copper–imidazole[8c,12] and copper–pyridine[13] complexes. The method treats the imidazole (or pyridine) ring as a structural unit and is able to give an accurate determination of the positions of all the ring atoms. Initial refinements were carried out on Fourier filtered data to reduce uncertainties caused by the limited signal to noise inherent in the experimental data. Essentially all the features of the EXAFS can be reproduced when all the atoms of two imidazole rings, coordinated at 2 Å, are included in the simulation together with the two O at 1.90 Å and the sulfur at 2.38 Å (Figure 4a). Four carbon atoms from imidazole are required at 2.91 Å. The intense fourth shell peak in the Fourier transform at 4 Å, which is predominantly due to multiple scattering interactions involving the outer shell of atoms of the imidazole rings,[12] is largely accounted for by the presence of the two nitrogen and two carbon atoms from imidazole at 4.14 Å.

As is evident from the fit to the Fourier transform in Figure 4a, the 4-Å peak is not completely simulated by the imidazole

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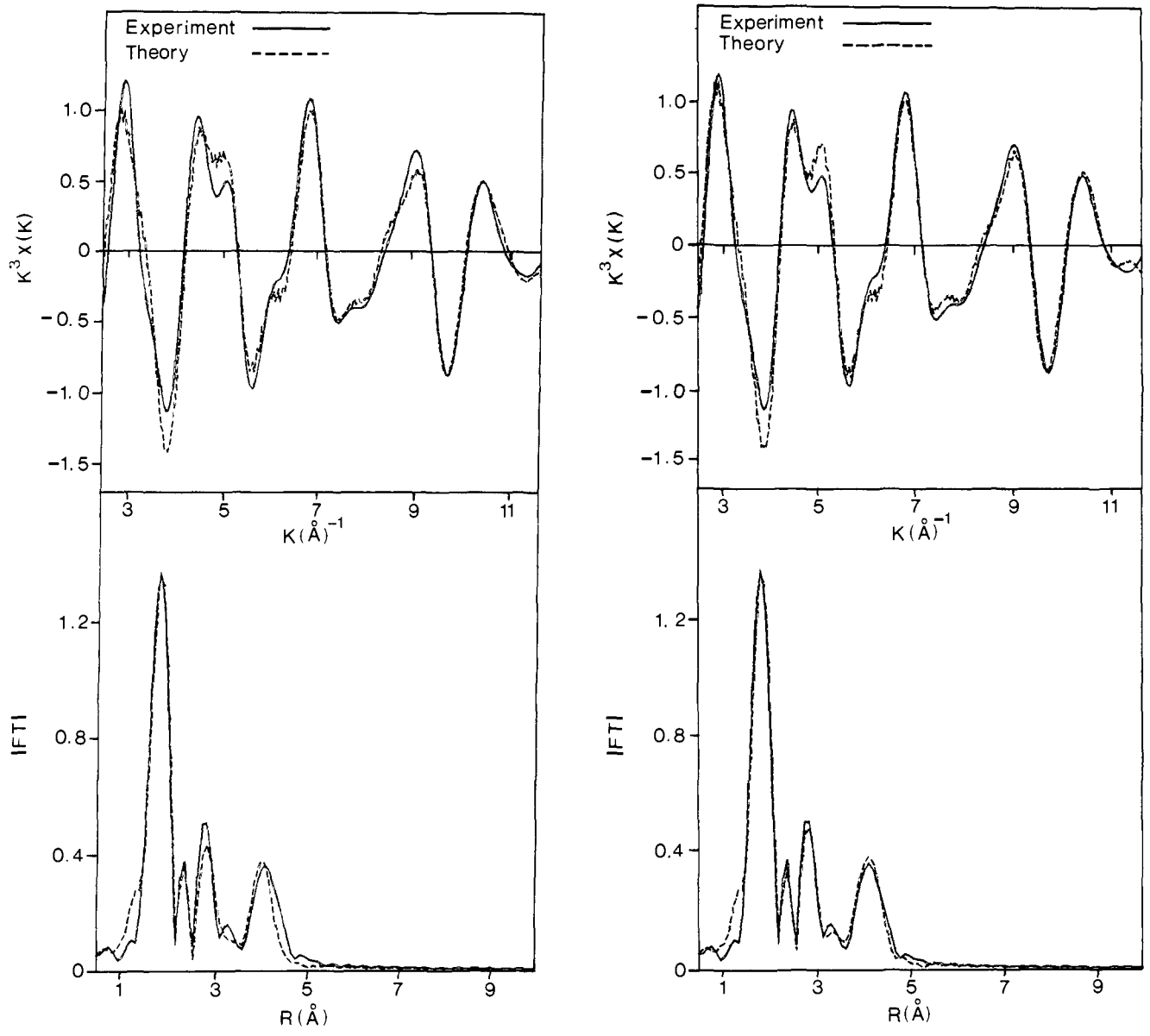


Figure 4. $K^3$-weighted simulation of the Fourier filtered experimental spectrum showing (a, left) the fit obtained using 2 imidazole rings coordinated at 2.00 Å with 2 oxygen atoms at 1.90 Å and a sulfur ligand at 2.38 Å and (b, right) the same fit with "extra" carbon atoms present (see text for details).

backscattering. Additional backscatterers seem to be required in the range 4.2–4.5 Å, and the fit to both the EXAFS and Fourier transform may be improved by including single scattering from three additional low-Z atoms at 4.3 Å. Further improvements in the goodness of fit also arise if additional carbon atoms are included at 2.97 and 3.16 Å (Figure 4b). This simulation is compared with the raw data in Figure 5. The fit parameters are shown in Table I. The source of these "extra" backscatters is not clear. The possibility that they are associated with a third coordinated imidazole is unlikely as 4.3 Å is substantially larger than would be expected for C3/N5 atoms of imidazole. Also, no multiple scattering is required for these atoms, which is a characteristic feature of imidazole atoms at these distances. One possibility is that these "extra" atoms arise from a phenolate-like ligand coordinated at 1.90 Å. Coordination of phenolate at 1.90 Å to $Cu^{2+}$ in a mixed phenolate–imidazole complex is known.[22] Some of these extra carbon atoms may originate from the peptide providing the ligands to copper.

## Discussion

The quality of simulation we have achieved for both the raw and Fourier filtered EXAFS data and their transforms is good, which gives confidence in the interpretation. Scott and Dooley[10] compared the EXAFS data for bovine plasma amine oxidase (BPAO) with that of $Cu(Imid)_4(NO_3)_2$ and concluded that the $Cu^{2+}$ sites in the native enzyme had 3–4 coordinated nitrogens (or oxygens) at 1.99 Å; this structural information was consistent with a model for the $Cu^{2+}$ sites having three histidine nitrogens and one water coordinated in the plane. We can now refine this model in several important ways. First, we have not been able to simulate the prominent peak at 2.38 Å in the Fourier transform unless we include a sulfur in the primary coordination sphere; the distance of 2.38 Å is more consistent with a thioether (e.g., from a methionine) than a thiolate.[21] Another possibility to explain this peak is that it comes from chloride as a ligand; this has been proposed as an alternative to methionine from EXAFS studies on the $Cu_B$ site in cytochrome *c* oxidase.[23]

There have been earlier claims that sulfur might be a coordinating ligand to Cu in BPAO based on the presence of CD bands in the 300–400 nm region.[24] Magnetic CD studies[25] indicated

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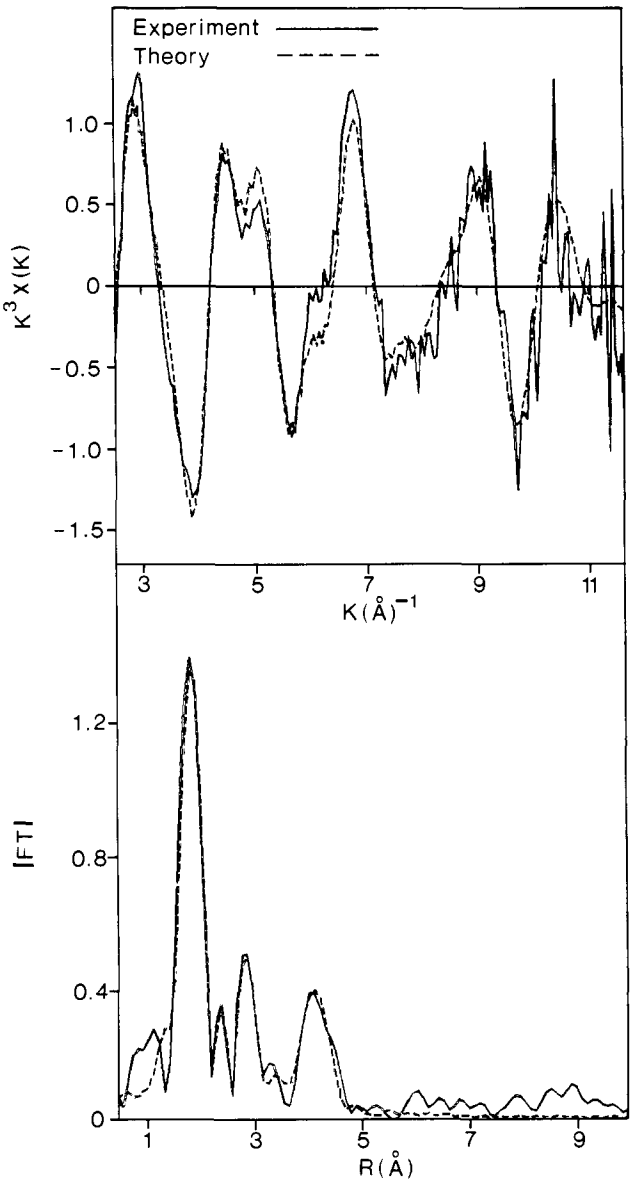


**Figure 5.** $K^3$-weighted simulation of the raw EXAFS data using the same parameters as in Figure 4b.

that the sulfur did not originate from thiolate and later studies comparing the spectral properties of native and apo forms of the enzyme concluded that there was no evidence for a S → Cu charge-transfer transition in the 300–400 nm region of the optical spectrum.[26] The conclusion from the present EXAFS studies for thioether (or chloride) as a ligand to Cu needs substantiation by other spectroscopic methods. It can be pointed out that the peak at 2.38 Å is also observed in the Fourier transform of the EXAFS spectrum of the "1-Cu" form of the enzyme (Figure 1a), which argues against sample artefacts being responsible.

The second refinement to the model is the evidence for two discrete distances within the first shell, namely Cu–O at 1.90 Å and Cu–N at 2.00 Å. Multiple scattering treatment shows that

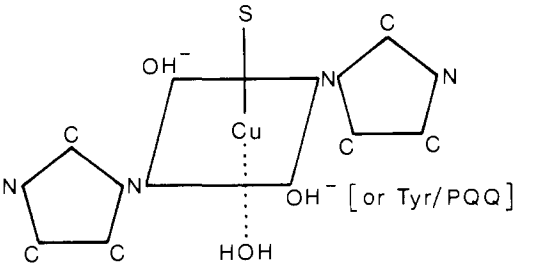


**Figure 6.** Postulated model for the Cu site in amine oxidase based on the EXAFS analysis and other spectroscopic observations.

the Cu–N distance is broadly consistent with two coordinated histidine imidazole ligands. There is no indication from the present EXAFS data for the coordinated imidazoles being chemically distinct. This conclusion has been drawn from electron spin echo (ESE) modulation studies on the amine oxidase from pig kidney and bovine plasma. Since ESE studies have not been carried out on pig plasma enzyme, ESE and EXAFS results are not necessarily inconsistent. The short distance at 1.90 Å from $Cu^{2+}$ to a first shell low-Z ligand is strongly indicative of equatorially coordinated hydroxyl rather than water (expected distance 2.10 Å) or nitrogen (expected distance 1.99 Å).[8b] Metal-bound hydroxyls are good nucleophiles, and this may be important in the catalytic mechanism as has been proposed earlier.[27] One of the two oxygens might originate from a coordinated tyrosine from the protein. It is interesting to speculate whether the EXAFS data provide evidence for association between the copper and PQQ cofactor in the active site of PPAO. F NMR relaxation studies on the complex between PPAO and fluorinated derivatives of the inhibitor phenylhydrazine indicate that direct interaction between copper and the carbonyl grouping of the organic cofactor is unlikely. However, it is argued that the cofactors might be in opposition. This could perhaps account for the "extra" atoms required in the present EXAFS simulations. Finally, it is clear that the copper centers in native (2-Cu) and 1-Cu forms of the enzyme are quite similar.

In summary, direct evidence for the environment of the Cu sites in amine oxidase has been provided by using the multiple scattering analysis of EXAFS data. The results clearly indicate two histidines and a heavy atom (thioether sulfur or chloride) as ligands. Evidence for two short Cu–O at 1.90 Å has been provided which is compatible with equatorial $OH^-$ or phenolate linkages. An additional ligand, such as water, may be present in the axial position at a distance >2.3 Å as indicated by water proton relaxation data[3] and ENDOR.[6] However, no evidence for this has been provided here. A possible model for the active site of PPAO consistent with the EXAFS data and earlier spectroscopic observations is shown in Figure 6.

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