Communications to the Editor

Sulfur 2p Photoelectron Spectrum of *Limulus* Oxyhemocyanin. Reply to Observations on the ESCA Spectra of Plastocyanins

Sir:

We have previously published sulfur 2p (S2p) photoelectron spectra of French bean (*Phaseolus vulgaris*) plastocyanin.[1] Signals at 169 and 164 eV were observed, and the one at high binding energy (HBE) was interpreted as direct evidence of Cu-S coordination in the protein. Recently, however, Peeling et al.[2] have challenged this conclusion. They claim that the plastocyanin HBE S2p signal is attributable to the presence of sulfate or some other form of oxidized sulfur impurity. In this note we emphasize that our previous results, combined with new data on *Limulus* oxyhemocyanin and reduced plastocyanin, eliminate impurity contamination as a possible explanation for the HBE signal we observed.

Peeling et al.[2] have failed to confront directly the evidence on which our interpretation was based. Central to this interpretation was the sequence of native, apo, and cobalt(II)-substituted plastocyanin spectra. A sample of native plastocyanin showed the two S2p peaks at 169 and 164 eV. An aliquot from this native sample was dialyzed against cyanide to remove the copper.[3] The resulting apoprotein showed no HBE S2p peak. However, the HBE S2p signal was restored almost to its original intensity by addition of cobalt(II) to the apoprotein sample. It is important to note that the same protein sample was carried through this sequence of copper removal and cobalt(II) reconstitution. Furthermore, it had already been established that cobalt(II) substitution occurs at the copper binding site in blue proteins.[3,4] The cobalt(II) solution used to reconstitute the apoprotein was an atomic absorption standard. No sulfate could be detected in this solution by standard analytical methods. Therefore, a sulfate impurity theory is not consistent with the disappearance of the HBE S2p peak in the apoprotein, nor does it account for the restoration of the signal at appropriate intensity upon cobalt(II) reconstitution.

A HBE S2p signal at 169 eV has been observed in the spectra of two other blue copper proteins, *Rhus vernicifera* stellacyanin and *Pseudomonas aeruginosa* azurin.[5] The HBE S2p signal disappears upon copper removal in stellacyanin and azurin, and it reappears with cobalt(II) reconstitution in stellacyanin. Studies on cobalt(II) azurin have not yet been completed.

We have also performed preliminary experiments which indicate that the HBE S2p signal is not present in the S2p spectrum of plastocyanin that has been reduced with ascorbic acid. The plastocyanin was prepared as previously described[3] and reduced under a nitrogen atmosphere by the addition of a 200-fold excess of ascorbic acid. Spectra of dried films were recorded at 200 K. As a control, sulfate was added to a protein solution containing a 100-fold excess of ascorbic acid; the sulfate S2p signal was not affected by the presence of ascorbic acid. Therefore, sulfate impurity theories are inconsistent with the reduced plastocyanin result.

Peeling et al.[2] reported the presence of a dialyzable oxidized sulfur impurity in their hemocyanin spectra. For comparative purposes, we have made spectral measurements on *Limulus* oxyhemocyanin that was isolated using low and high speed ultracentrifugation. No sulfate was employed in the isolation, but no special precautions were taken to avoid impurities. The S2p spectrum from a sample of this hemocyanin preparation is shown in Figure 1. There is only a trace of a HBE S2p signal, which contrasts with the minimal 0.9:1 ratio of high to low binding energy peaks observed by Peeling et al.[2] We conclude, therefore, that an oxidized-sulfur impurity was present in the preparations of Peeling et al.[2]

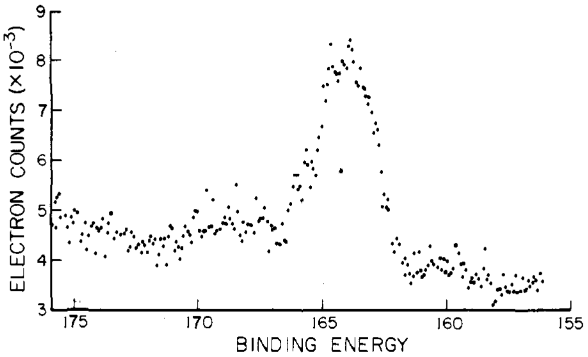

Figure 1. Sulfur 2p photoelectron spectrum of *Limulus* oxyhemocyanin at 200 K. A low energy electron flood gun set at 0.05 mA and 1.5 eV was used to control charging. Binding energy was corrected to aliphatic carbon 1s at 285.4 eV.

In conclusion, impurity theories do not account for the systematic appearance of the HBE S2p signals we have observed. In stellacyanin the HBE S2p signal must be associated with cysteine sulfur coordination to Cu(II) (or Co(II)), as this protein contains no methionine residues.[6] It is reasonable to assume that the HBE S2p signals in the spectra of plastocyanin and azurin are also attributable to active-site cysteine sulfur coordination. The results on oxyhemocyanin demonstrate our ability to prepare protein samples free of oxidized-sulfur species, in contrast to the impurity problem reported by Peeling et al.[2] The absence of a significant HBE S2p signal in the spectrum of oxyhemocyanin is consistent with the fact that several independent chemical[7-10] and vibrational-spectroscopic[11] studies have uncovered no evidence whatsoever for cysteine sulfur ligation at the copper site in various hemocyanins.

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References and Notes

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Spectral Studies of Octamethyldimetalates of Molybdenum(II), Rhenium(III), and Chromium(II). The Assignment of the δ → δ* Transition[1]

Sir:

Two recent theoretical studies[2,3] of the quadruply bonded octachlorodimetalates $Mo_2Cl_8^{4-}$ and $Re_2Cl_8^{2-}$ have suggested that the lowest energy visible absorption band for each complex (~1.88 and ~1.42 μm^{-1}, respectively) be assigned as the electric dipole allowed $b_{2g}\delta \rightarrow b_{1u}\delta^*$ ($^1A_{2u} \leftarrow {}^1A_{1g}$) transition. The δ and δ* orbitals are symmetry determined as $\delta = [d_{xy}(1) + d_{xy}(2)]/2^{1/2}$ and $\delta^* = [d_{xy}(1) - d_{xy}(2)]/2^{1/2}$ and are the highest filled and lowest unoccupied molecular orbitals. The polarized crystal spectrum[4,5] of $[(C_4H_9)_4N]_2Re_2Cl_8$ has confirmed this assignment for the rhenium(III) dimer. Entirely complementary results come from the resonance Raman experiments[6] on this compound and its bromo analogue. Corroborating evidence for a similar assignment of the 1.88-μm^{-1} band in salts of the dimolybdenum ion rests on the observation[7] of resonance Raman effects when these complexes are irradiated with 514.5 Ar^+ laser excitation, a wavelength close to the absorption maxima.

Although primarily metal d in character, the δ and δ* orbitals interact with halide pπ orbitals of identical symmetry. Group theoretical considerations, based on D_{4h} molecular symmetry, establish the existence of b_{2g} and b_{1u} ligand π orbitals, and SCF-XαSW calculations of ground state valence energy levels reveal that these interactions may be considerable. Norman and Kolari[2] calculate for $Mo_2Cl_8^{4-}$ that $b_{2g}\delta$ and $b_{1u}\delta^*$ are 11 and 7% halide in character. For $Re_2Cl_8^{2-}$ these values increase to 21 and 18%, respectively, and may be even higher.[3] The higher formal oxidation state of rhenium may in part account for this behavior. It is clear that a discussion of δ → δ* transitions in these two dimers must recognize the influence of metal-halide π bonding.

One way to alleviate the effect of this M-L π bonding on the orbitals is to study closely related complexes where this bonding is reduced due to the intrinsic properties of the ligand. We report here some observations on the electronic spectra of the octamethyldimetalates of chromium(II), molybdenum(II), and rhenium(III), the first isoelectronic series which encompasses all three transition metal series. Inasmuch as the methyl ligand meets the requirement specified above, these complexes afford an excellent opportunity to study the δ → δ* transition as a function of the metal atoms without complications due to varying degrees of metal-ligand π interaction. In the methyl dimers, $M_2(CH_3)_8^{n-}$, the δ orbitals become essentially 100% metal in character. Thus the δ → δ* transition should reflect the extent of metal-metal overlap,[8] although (as discussed below) an increase in metal-metal δ overlap does not necessarily imply observable M-M bond shortening.

Table I. ^1H and $^{13}C\{^1H\}$ NMR Parameters for $M-CH_3$[a]

Complex	δ^1H	$\delta^{13}C$
$[Li(Et_2O)]_4Cr_2(CH_3)_8$	−0.47	+9.60
$[Li(Et_2O)]_4Mo_2(CH_3)_8$	−0.30	+4.71
$[Li(Et_2O)]_2Re_2(CH_3)_8$	+0.71	—
$[Li(THF)]_2Re_2(CH_3)_8$	—	+16.06

[a] All complexes were run as solutions in benzene-d_6. Proton and carbon-13 chemical shifts were adjusted to the Me_4Si scale using the known chemical shifts for deuteriobenzene (+7.24 and +128 ppm, respectively). Proton NMR were run on a Varian Associates A60-A spectrometer; Fourier transform carbon-13 NMR were run on a Varian Associates XL-15FT spectrometer operating at 25.16 MHz.

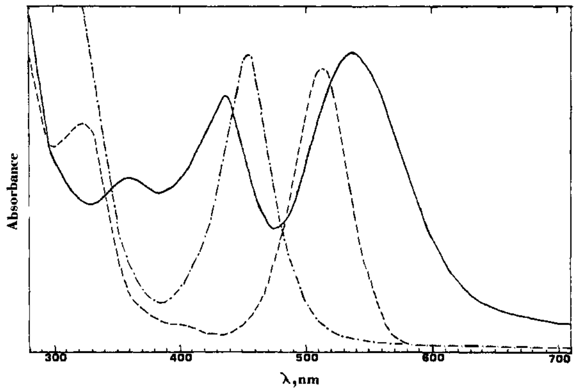

Figure 1. Electronic spectra of $Re_2(CH_3)_8^{2-}$ (—), $Mo_2(CH_3)_8^{4-}$ (- - -), and $Cr_2(CH_3)_8^{4-}$ (- · · -) in diethyl ether. Band maxima are presented in Table II. The ordinate is not drawn to scale (see note *c* in Table II).

The chromium[9] and molybdenum[10] complexes were prepared by literature methods and characterized by NMR (Table I) and/or metal analysis. The rhenium complex was prepared from $Re_2(CH_3COO)_4Cl_2$ and methyl lithium in diethyl ether at 0 °C. Metal analysis and NMR are consistent with the formulation $[Li(Et_2O)]_2Re_2(CH_3)_8$. All three complexes are exceedingly sensitive to air and moisture. The crystal structures of the group 6 complexes have been reported.[10,11] The molecular units possess structural and magnetic properties consistent with their formulation as quadruply bonded complexes. While pursuing these studies, we became aware of the independent preparation of the methyl rhenium dimer by two other groups.[12] A structural characterization consistent with δ-bond formation has been presented.

For UV-visible spectrophotometry an all-glass greaseless apparatus was constructed so that reactions between metal precursors and methyllithium could be performed in situ and filtered and the spectra of complexes recorded without the necessity of solution transfers.[14] The spectra are presented in Figure 1 and band positions are listed in Table II. No other band maxima were observed between the wavelengths of 230 and 2000 nm. All spectra were reproduced at least three times.

The spectrum of $Mo_2(CH_3)_8^{4-}$ emphasizes a point made earlier. It is qualitatively very similar to that of $Mo_2Cl_8^{4-}$.[7] There is a slight blue shift of the δ → δ* transition from ~1.88 to 1.95 μm^{-1} which we attribute to an increase in metal-metal δ overlap concomitant with removal of 7-11% ligand character in the δ orbitals. That this small increase in overlap does not result in any observable decrease in bond length is evident from