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Some Observations on the ESCA Spectra of Plastocyanins

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Abstract: The core level spectra of a number of plastocyanins have been investigated by means of ESCA. The higher binding energy component in the S_{2p} levels observed for particular samples is shown to arise from extraneous sulfur present in a high oxidation state rather than from coordination of sulfur-containing amino acids to the metal ion, as has previously been claimed.

Solomon et al.[2a] have recently reported the results of an ESCA study on the "blue" copper protein plastocyanin. A single peak at 164 eV binding energy in the S_{2p} region of the spectrum was observed for the apoprotein, while an additional peak shifted by about 5 eV to higher binding energy was observed in that of the Cu and Co proteins. The bean (*Phaseolus vulgaris*) plastocyanin studied, contains two methionine and one cysteine[12] residues,[2b] and since the ratio of the intensity of the S_{2p} peak at higher binding energy to that at the lower binding energy component was about 1:2, the spectra were interpreted as indicating that one of the sulfur atoms, probably that of the single cysteine residue, binds to the metal atom in the plastocyanin.

We have used the ESCA technique to examine a number of proteins including the copper proteins, plastocyanin, and hemocyanin; our results and those of a number of previously published studies lead us to suggest that the higher binding energy component is due to sulfate or some other form of oxidized sulfur present in the metalloprotein preparations. ESCA is essentially a surface technique (the mean free paths through a solid of electrons photoemitted by Al or Mg Kα x rays being of the order of a few tens of ångströms or less). Fur-

Table I. Experimental S_{2p} Binding Energies of Various Compounds

Sample	S_{2p} binding energy[a]	
	A	*B*
Na_2SO_4		168.3 ± 0.1
Sulfoamino acids[6]	163.3 ± 0.1	
Proteins reported in ref 6	163.4 ± 0.2	167.7 ± 0.3
Ovalbumin	163.5 ± 0.1	167.4 ± 0.2
Cytochrome *c*	163.2 ± 0.1	167.6 ± 0.2
Hemocyanin (a) as received	163.6 ± 0.2	168.4 ± 0.3
(b) dialyzed	163.7 ± 0.2	167.8 ± 0.3
Plastocyanin, average	163.3	167.3
Potato, native	163.3 ± 0.2 (1)	167.4 ± 0.4 (0.3)
apoprotein	163.2 ± 0.2	
Cytisus	163.2 ± 0.1	
Ground elder	163.3 ± 0.2 (1)	166.9 ± 0.4 (0.8)
Marrow	163.2 ± 0.2 (1)	167.8 ± 0.3 (0.9)
Giant hogweed, native	163.3 ± 0.2 (1)	167.1 ± 0.3 (0.6)
apoprotein	163.2 ± 0.2 (1)	167.4 ± 0.3 (1)
Clover	163.2 ± 0.1 (1)	167.2 ± 0.3 (0.35)

[a] A = low-binding energy component. B = high-binding energy component. The values in parentheses for the plastocyanins are the relative intensities of the two peaks.

thermore, as the S_{2p} lines in these proteins are relatively weak, a minute amount of impurity could give rise to the second S_{2p} peak. Indeed, the possibility of interference from impurities, particularly in studies of weaker signals in samples of biological interest, has been stressed before.[3]

Results and Discussion

In a study of non-heme iron proteins Kramer[4] showed that two lines in the lower binding energy region (163.0 ± 0.2 and 161.4 ± 0.2 eV) corresponded to the presence of cysteine and acid-labile sulfur, while a peak at higher binding energy was assigned to an oxidized sulfur impurity;[4] the presence of a peak due to oxidized sulfur in a number of iron proteins was confirmed in a more recent study.[5] The peak persisted even though the proteins were extensively desalted and the impurity (assumed to be sulfate) appeared to be more tightly bound to some proteins than to others. This same study reported an investigation by ESCA of a number of copper proteins. The S_{2p} spectra of these proteins also contained a peak at high binding energy "attributed to sulphate or some other form of oxidized sulfur".[5] Similarly, we have observed that oxidized sulfur gives a peak ~4 eV higher in binding energy than the amino acid sulfur in a number of proteins and that on oxidizing all the sulfur in a protein, the S_{2p} peak shifts by about 4.7 eV to higher binding energy.[6] A similar shift was observed by Siegbahn et al.[7] in a study of the oxidation of insulin monitored by ESCA. It is, therefore, apparent that the presence in proteins of a S_{2p} component having a binding energy of about 167–169 eV is indicative of the presence of some form of oxidized sulfur.

In addition, the available evidence suggests that coordination of sulfur to a transition metal results in a relatively small decrease in the S_{2p} binding energy rather than the large increase suggested by Solomon et al.[2a] Thus, Kramer found that acid-labile sulfur in non-heme iron proteins had a binding energy 1.6 eV lower than the cysteine sulfur, and further that the S_{2p} binding energy of cysteine sulfur coordinated to the iron was indistinguishable from that of other cysteines in these proteins.[4] The S_{2p} peaks of model compounds in which sulfur is coordinated to iron were also lower in binding energy than uncoordinated protein sulfur. Iron proteins and a number of model compounds containing Fe–S coordination were also studied by Grunthaner,[5] and his results confirmed that labile sulfur coordinated to Fe was lower in binding energy than cystine sulfur; in addition, cystine sulfur coordinated to a metal was slightly lower in binding energy than unbound cystine. Grunthaner also examined by ESCA some copper proteins and a number of model compounds in which sulfur coordinates to Cu. For both the model compounds and the proteins, sulfur bonded to copper had a lower binding energy than free cystine or cysteine sulfur (by about 1.1 eV in the case of the proteins), and sulfur present as sulfide, $R-S^-$, was still lower in binding energy. Finally, the effect of sulfur coordination to mercury has been examined by Millard,[3] who studied the ESCA spectra of some model Hg–S compounds and some fairly well characterized Hg-protein derivatives. The S_{2p} binding energies of mercury dibenzyl mercaptide and Hg–S were lower than those of sulfur in proteins, but for the Hg proteins the S_{2p} line did not shift appreciably, indicating that the shift on coordination to the Hg is probably small. It appears on the basis of these studies that a shift in the S_{2p} peak of about 5 eV to higher binding energy on coordination to a metal is not to be expected.

In fact, Lindberg et al.[8] have examined the S_{2p} spectra of a large number of compounds and found an excellent correlation to exist between the S_{2p} binding energy and a semiempirical charge parameter estimated from electronegativities and the partial ionic character of bonds involving the sulfur. Using their correlation an increase in S_{2p} binding energy of 5 eV demands a net loss of 1 unit negative charge from the sulfur. In our view, it is not reasonable to expect such a change to occur simply on coordination of the sulfur to a copper atom. In the extensive series of sulfur compounds studied by Lindberg et al.[8] a shift of this magnitude was found only when the sulfur was bonded to highly electronegative elements.

Our own studies on a number of proteins are consistent with the assignment of the S_{2p} peak at high binding energy to an oxidized form of sulfur. From the results of Table I a peak at high binding energy was observed, not only for the copper proteins, hemocyanin and plastocyanin, but for a variety of other proteins as well. Furthermore, for the different varieties of native plastocyanin studied the relative intensity of the S_{2p} peak at higher binding energy to that of the lower binding energy component was found to be as high as 0.9:1 in the case of marrow plastocyanin, while for Cytisus plastocyanin no high binding energy component of significant intensity was observed. The apoprotein from potato plastocyanin showed only one S_{2p} peak, but that derived from giant hogweed plastocyanin had two peaks of approximately equal intensity in this region

of the spectrum. Representative ESCA spectra of the S_{2p} which illustrate this point are shown in Figure 1. It is evident from these data that the appearance of two peaks in the S_{2p} region of the spectrum is not unique to the metalloproteins, while the apoprotein may, in fact, yield such a spectrum. These facts cannot be reconciled with the interpretation put forward by Solomon et al.[2a] for the spectra they reported.

It should be noted that the high-binding energy peak in the S_{2p} spectrum of the proteins was the broader of the two, due perhaps to the presence of a number of different forms of oxidized sulfur or to the effects of different lattice potentials on the binding energies of different sulfur atoms in the samples. For this reason uncertainties in the measured binding energies for this component are generally somewhat larger than for the peak at lower binding energies.

From a consideration of the relative intensities of the N_{1s} and S_{2p} peaks it is possible to verify that all the protein sulfur (that is methionine, cystine, and cysteine residues) in the plastocyanins contributes to the peak at 163.2 ± 0.2 eV and hence that the high-binding energy peak is due exclusively to oxidized sulfur. Thus, from the ESCA spectra of a number of model compounds containing both N and S the relative N_{1s} to S_{2p} photoelectric cross section for our spectrometer was found to be 0.66 ± 0.6. It has been shown before, both in our studies[6] and by Millard,[3] that the relative intensities of the N_{1s} to S_{2p} peaks in the ESCA spectra of proteins are proportional to the N/S content of the proteins. We can, therefore, use the experimental intensity ratios of the N_{1s} and S_{2p} peaks to calculate the N/S stoichiometry for the plastocyanin samples examined. When the high-binding energy component of the S_{2p} spectrum is neglected the calculation using experimental data gives an average N/S stoichiometry of 39.5 ± 5.0 for the plastocyanins studied. Using the complete amino acid sequence data for potato[9] and marrow[10] plastocyanins, the theoretical N/S stoichiometry is found to be 39.3. If the high-binding energy component of the S_{2p} spectrum is included in the calculation, the calculated experimental stoichiometry varies from 17.7 to 28.8 with different plastocyanins. This evidence suggests strongly that only the protein sulfur contributes to the S_{2p} peak of binding energy 163.3 eV; there is no support for the suggestion advanced by Solomon et al.[2a] that part of the protein sulfur contributes to a peak at higher binding energy.

The possibility of detecting S–Cu coordination in "blue" copper proteins via large shifts in the S_{2p} electron binding energy of the ESCA spectrum was investigated using hemocyanin. Again two S_{2p} peaks appeared, with binding energies of 168.4 and 163.6 eV. The relative intensities of these peaks were 1.9:1. On dialysis of the protein, the intensity of the high-binding energy component was substantially reduced, giving a final value of 0.9:1 for the relative intensities of the two S_{2p} peaks. It is interesting to note that the centroid of the high-binding energy peak, which was quite broad, decreased in apparent binding energy to 167.8 eV on dialysis. Comparing the binding energies in the two samples to that of sulfur in Na_2SO_4 it appears that dialysis probably removes a substantial amount of SO_4^{2-}, but oxidized sulfur of some other form remains, either in the form of a surface contaminant or as some form tightly bound to the protein.

The ratio of the intensity of the N_{1s} peak to that of the S_{2p} peak of hemocyanin was measured in order to calculate the N/S stoichiometry and to determine whether any protein sulfur contributes to the high-binding energy S_{2p} component. Using only the S_{2p} peak at 163.6 eV, the N_{1s}/S_{2p} intensity ratio was the same before and after dialysis and led to a calculated N/S stoichiometry of 32 ± 5. If the total intensity of both sulfur peaks was used in the calculation the apparent N/S stoichiometry before dialysis was 10.9 ± 1.8 and after dialysis 17.6 ± 2.6. N/S stoichiometry calculated from the amino acid composition gave the value of 34.48 ± 1.63, which is in good agreement with ESCA data using the S_{2p} peak at 163.6 eV only.

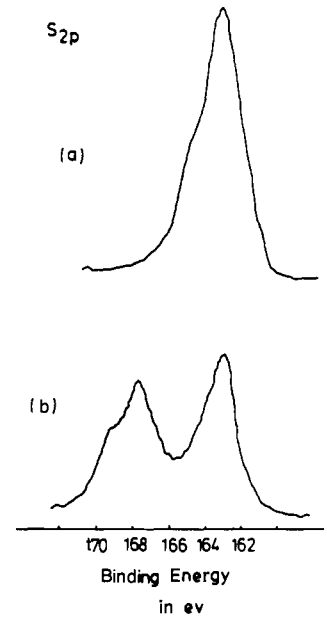

Figure 1. Typical S_{2p} levels for (a) cytisus plastocyanin representative of a metallo protein system showing only a low-binding energy S_{2p} region, and (b) the apoprotein of giant hogweed plastocyanin representative of a decoppered metallo protein system exhibiting both high- and low-binding energy regions.

In conclusion, we have not found any evidence for S–Cu coordination in plastocyanin from the ESCA studies. This does not preclude the possibility that such coordination occurs, but if it does the resulting shift in the S_{2p} binding energy is small. This expectation is confirmed in several studies including the results obtained with hemocyanin. The high-binding energy S_{2p} peak which Solomon et al.[2a] attributed to sulfur coordinated to Cu arises from the presence in the protein samples of sulfate or some other form of oxidized sulfur.

Experimental Section

ESCA spectra were recorded on an AEI-ES200B Electron Spectrometer using Mg $K\alpha_{1,2}$ radiation and were essentially identical in appearance to those obtained with a monochromatic Al $K\alpha_{1,2}$ source of radiation. Since signal accumulation time was much shorter using the unmonochromatized Mg x rays (about 15–20 min per spectrum), this radiation source was used throughout. The samples were checked for radiation damage by visual inspection and by monitoring the appearance of the C_{1s}, O_{1s}, N_{1s}, and S_{2p} regions over the time required to obtain the spectra.

Protein samples were either in the form of powders mounted on the spectrometer probe tip by double-sided tape or thin films cast from aqueous solution onto scrupulously clean gold foil, which was then fastened to the probe tip. In the case of hemocyanin and most of the plastocyanins, spectra were obtained on samples in both forms and no significant differences were found. For hemocyanin and the plastocyanins the spectra were obtained at a temperature of 230–240 K.

Binding energies were determined relative to the hydrocarbon-type C_{1s} signal at 285.0 eV. Peak areas were measured using the electronic integrator of a Dupont 310 Curve Resolver.

Hemocyanin from *Megathura cremulata* (keyhole limpet), grade A, was obtained from Calbiochem (King Street, Hereford HR4 9BQ). Plastocyanin from potato (*Solanum tuberosum*), broom (*Cytisus ballendieri*), ground elder (*Aegopodium podagraria*), marrow (*Cucurbita pepo*), giant hogweed (*Heracleum mantegazzianum*), and clover (*Trifolium medium*) were purified as in Ramshaw et al.[11] Other proteins were obtained from Sigma Chemicals (Norbiton, Station Yard, Kingston-upon-Thames, Surrey).

Proteins were extensively dialyzed against deionized water and after dialysis half the protein solution was freeze dried and the other half

left in the aqueous state. Hemocyanin was hydrolyzed in 6 M HCl (constant boiling) at 110 °C for 22 h in an evacuated sealed tube. The hydrolyzates were prepared in duplicates. Cyst(e)ine content was determined as cysteic acid, following oxidation with performic acid.

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^{13}C NMR Relaxation Studies of Complexes between *cyclo*(L-Pro-Gly)$_3$ and Amino Acids. Conformational Aspects of Stepwise Binding†[1]

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Abstract: ^{13}C NMR relaxation and chemical shift measurements have been used to study the complexes of proline and valine amino acid salts (HCl·Pro-OBz and HCl·Val-OMe) with the synthetic ion-binding cyclic hexapeptide, *cyclo*(L-Pro-Gly)$_3$. While HCl·Pro-OBz was found to form a discrete 1:1 complex with the cyclic peptide, HCl·Val-OMe forms a 2:1 complex, an "amino acid sandwich", as well as a 1:1 complex. Evidence from appropriate changes in NT_1 values and ^{13}C chemical shifts confirmed the existence of these various complexes; for example, the NT_1 values found for α-carbon atoms of the free amino acid, HCl·Pro-OBz, and the free peptide decreased in the complexes to values which were identical within experimental error. By observation of selective broadening of ^{13}C resonances upon binding the paramagnetic Mn^{2+} cation, the primary binding site of *cyclo*(L-Pro-Gly)$_3$ is inferred to be the Gly carbonyl groups.

Complexes between "larger" and "smaller" molecules, such as the binding of an enzyme to its substrate, are widespread in chemistry and biology.[2] In the larger moiety, binding is often accompanied by induced structural alterations which produce cooperative and/or allosteric phenomena.[3-5] Direct observation of the conformational adjustments of an enzyme, induced upon binding a substrate or cofactor, has been limited by the low molar concentrations obtainable and the complexities of proteins. Consequently, it is often difficult to probe the restrictions to motion, the detailed stoichiometry, and mutual conformational adjustments which occur in both components upon binding. Since aspects of molecular motion may be examined through ^{13}C spin-lattice relaxation measurements (T_1's),[6-11] while changes in molecular conformation of interacting species can be correlated with variations in chemical shifts,[12-14] ^{13}C nuclear magnetic resonance (NMR) spectroscopy is particularly well-suited for investigation of binding interactions.[15] These circumstances led us to apply ^{13}C NMR spectroscopic methods to a system in which aspects of conformation and molecular motion could be elucidated for both the larger and smaller components of a molecular complex.

In this communication we report the use of ^{13}C NMR to monitor the conformational changes which take place in the formation of complexes between a synthetic ion-binding peptide and certain amino acid salts. The stabilizing interactions involved in binding include, primarily, delocalization of the net positive charge of the amino acid ammonium group over the peptide carbonyl oxygens, as well as possible hydrogen bonding and hydrophobic interactions similar to those expected to stabilize enzyme:substrate or hormone:receptor complexes.

Impetus for the present investigation was provided by the discovery that amino acid ester hydrochlorides (which may be viewed as alkylammonium salts; e.g., $RNH_3^+Cl^-$) bind specifically to the cyclic hexapeptide, *cyclo*(L-Pro-Gly)$_3$[13] (designated hereafter as *c*(PG)$_3$)[16]. The cation binding capabilities of this peptide have already been described.[17] (We propose the trivial name "aminophore" to describe the class of compounds, including the present ion-binding cyclic peptide, capable of interacting specifically with and/or transporting compounds containing protonated amino groups.)

In contrast to metal ion: ionophore complexes, both components of amino acid:cyclic peptide complexes contain carbon atoms (and are of suitable molecular size) such that detailed structural features in both species can be studied spectroscopically by ^{13}C NMR. If a discrete amino acid:*c*(PG)$_3$ complex is formed (see Figure 1), its existence would be confirmed by the decrease in the average relaxation times, T_1, of the carbon atoms of both the peptide and the amino acid (relative to the T_1 values of the free components). A similar decrease of T_1's of the protons of acetylcholine was reported recently upon complex formation between ATP and acetylcholine,[18] and in a related approach, binding of ^{13}C-enriched ox-

† This paper is dedicated to Professor R. B. Woodward on the occasion of his sixtieth birthday. E.R.B. is pleased to acknowledge over thirty years of stimulating scientific and personal association.