X-Ray Photoelectron Spectra of Iron–Sulphur Proteins

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The X-ray photoelectron spectra of the 2p, 3s and 3p levels of iron in oxidized *Clostridium pasteurianum* ferredoxin indicate that the eight iron atoms in the molecule are indistinguishable. Their magnetic state is indicated both by core polarization splitting of the 3s electrons, and by 'shake-up' satellites on the 2p lines. Similar satellites are observed in the 2p lines of reduced *Chromatium* high-potential iron–sulphur proteins and oxidized spinach ferredoxin, indicating that there too the iron atoms are magnetic. The low observed magnetic susceptibility of these proteins is therefore due to spin-coupling between the iron atoms in the active centre.

We report measurements of magnetic effects in the X-ray photoelectron (XPS or ESCA) spectrum of iron atoms in some iron–sulphur proteins in the oxidized state.

A considerable number of magnetic measurements (e.p.r., ‡ p.m.r., Mössbauer effect, magnetic susceptibility) has been made in recent years on the iron–sulphur proteins (Palmer, 1973; Hall *et al.*, 1974). These molecules generally contain one or more active centres each consisting of a group of either two or four iron atoms tetrahedrally co-ordinated to sulphur atoms (Adman *et al.*, 1973). The sulphur tetrahedra are packed together so as to share common edges, and this structure provides superexchange paths between the iron atoms via the intervening sulphur atoms, so that the iron magnetic moments are antiferromagnetically coupled together.

We have studied two iron–sulphur proteins containing four-iron centres and one which has a two-iron centre. They are ferredoxin from the bacterium *Clostridium pasteurianum*, the high-potential iron–sulphur protein from *Chromatium*, and ferredoxin from spinach. *Clostridium* ferredoxin contains two four-iron centres and has a negative redox potential; high-potential iron–sulphur protein has one four-iron centre and a positive redox potential, and spinach ferredoxin has a two-iron centre and a negative redox potential. In the normal state (i.e. oxidized ferredoxin or reduced high-potential iron–sulphur proteins) the molecules have a non-magnetic ground state, i.e. they do not give an e.p.r. signal nor do they show a magnetic hyperfine interaction in their Mössbauer spectra. The Mössbauer-effect chemical shifts are the same for oxidized *Clostridium* ferredoxin (Thompson *et al.*, 1974) and reduced high-potential iron–sulphur

protein (Dickson *et al.*, 1974), and suggest that the active centres of both molecules contain formally two Fe^{3+} and two Fe^{2+} atoms. For oxidized spinach ferredoxin the Mössbauer chemical shift suggests that the molecule contains two Fe^{3+} atoms (Rao *et al.*, 1971).

The absence of a magnetic moment in all these molecules is believed to be due to antiferromagnetic coupling between the atoms, but very few measurements sensitive to the magnetic state of the iron have been made on the oxidized protein. P.m.r. (Poe *et al.*, 1970) showed shifts to both higher and lower frequencies with increase of temperature, which provided indirect evidence that there are magnetic atoms in the molecule which are spin-coupled. The present measurements were made on the iron atoms themselves and the data showed directly that they are individually in magnetic states.

The principles of photoelectron spectroscopy have been described by Siegbahn (1973) and by Shirley (1973).

In this technique a monochromatic beam of X-rays is allowed to impinge on a specimen; from which electrons are expelled. The kinetic energy of these electrons, which is measured by electrostatic deflection, is equal to the energy of the X-ray photon, minus the binding energy for the atomic level from which it is expelled. It is therefore possible to obtain a spectrum of the binding energies of all the electron levels of all the elements present in the specimen. Differences in the chemical states of an element are observed as small but detectable differences in the binding energy of the valence electrons. A limitation of the technique for biochemical purposes is that aqueous solutions cannot be studied, because the spectrometer works under a high vacuum. However, such samples can either be freeze-dried, or applied as a

‡ Abbreviations: e.p.r., electron paramagnetic resonance; p.m.r., proton magnetic resonance.

solution and evaporated in a suitable holder. The sensitivity of the apparatus has been developed to a stage where it is possible to measure contributions to the spectrum from elements present in low concentrations, such as transition metals in metalloproteins. Kramer & Klein (1972) have reported measurements of the binding energies of the 3p electrons of iron and also 2p electrons of sulphur in four different iron-sulphur proteins using this technique. Liebfritz (1972) has also reported data on photoemissions from the Fe 2p_{3/2} level in two iron-sulphur proteins.

In the experiments reported here the 2p and 3p photoelectrons of iron have been observed for the three proteins. The 3s photoelectrons of Fe were also observed for *C. pasteurianum* ferredoxin, and the spectrum showed a splitting due to core polarization which shows that the iron atoms are magnetic. This is also suggested indirectly by the observations of high-binding-energy ('shake-up') satellites on the 2p lines. These data confirm that the previously observed low magnetic susceptibility (e.g. Blomstrom *et al.*, 1964) arises from antiferromagnetic coupling between groups of iron atoms in the molecule.

Experimental

We have measured photoemission in the region of the 2p, 3s and 3p lines of iron by exciting with AlK α X-radiation [with energy 2.382×10^{-16} J (1486.6 eV)] in an AEI ES 100 spectrometer. The proteins were prepared as previously described (Rao *et al.*, 1971; Thompson *et al.*, 1974). The protein was buffered with 10 mM-Tris-acetate, pH 8.2, to avoid the interfering lines due to phosphorus and chlorine found in the photoelectron spectrum when phosphate or Tris-HCl buffers were used. Care was taken to avoid the use of silicone grease in the preparation, since silicon

lines occur close to the iron 3s lines in the spectrum. The proteins were used in the dried form and were introduced into the spectrometer in two different ways: (i) as a freeze-dried powder and (ii) as a freshly prepared aqueous solution which was immediately dehydrated by pumping on it inside the spectrometer chamber. The two methods gave identical spectra, and the binding energy observed for the Fe 3p level agreed closely with previous measurements made on frozen aqueous solutions (Kramer & Klein, 1972).

Results

(i) Oxidized *Clostridium ferredoxin*

Clostridium ferredoxin has a molecular weight of 6000 and contains eight iron atoms per molecule. Iron 2p, 3s and 3p lines were observed for this protein and the binding energies of these levels are shown in Table 1. The line-widths are comparable with those found in ionic iron salts. The lines of Fe³⁺ and Fe²⁺

Table 1. Electron-binding energies (in eV) in photoelectron spectra of iron in some iron-sulphur proteins

Measurements were made as described in the Experimental section. The uncertainty is estimated to be ± 0.2 eV.

Level	<i>Clostridium</i> ferredoxin (eV)	<i>Chromatium</i> high-potential iron-sulphur protein	Spinach ferredoxin
2p _{1/2}	721.9	721.1	721.2
2p _{3/2}	708.7	708.0	708.5
3s _↑	97.6	—	—
3s _↓	92.8	—	—
3p	55.1	54.1	54.6

Fig. 1. Photoelectron spectrum of oxidized *Clostridium ferredoxin* in the Fe 3s region

The line on the left is probably due to silicon impurities. Spectra were recorded as described in the Experimental section.

typically differ in energy by 2 eV (Buchanan *et al.*, 1971) and so can be distinguished in photoelectron spectra. The spectra of oxidized *Clostridium* ferredoxin show no detectable differences in the valence states of the iron atoms, in agreement with the Mössbauer-effect data (Blomstrom *et al.*, 1964; Thompson *et al.*, 1974).

If the iron atoms in ferredoxin are magnetic, the energy of the filled core electron levels (especially the 3s state) will be split by exchange interaction with the unpaired 3d electrons (Fadley *et al.*, 1969). Further, this splitting will not be affected by the magnetic coupling between different atoms. Hence X-ray photoelectron spectroscopy should enable the magnetic state of the iron atoms to be confirmed directly. Fig. 1 shows the spectrum of the 3s level. It is split, apparently into three peaks at binding energies (uncorrected for charging effects, work function etc.) of 102.1, 97.6 and 92.8 eV. The line at 102.1 eV was variable in intensity and its energy is that expected for the silicon 2p line, and hence we ascribe it to the presence of a silicon impurity. Moreover, its intensity correlated with that of the silicon 2s line at 149 eV, thus confirming this assignment. It is presumed that the silicon originates from silicone grease, but although the contaminant was considerably decreased by avoiding use of this grease it was not found possible to eliminate it entirely. The remaining two lines are ascribed to the 3s levels of iron; the lower-energy one is due to $3s_{\uparrow}$ electrons and lies 4.8 eV below the $3s_{\downarrow}$ line. This splitting arises from exchange interaction between the 3d and 3s electrons and shows that there are unpaired 3d electrons on the iron atoms. It also shows that the 3d electrons are not delocalized to molecular orbitals extending over the whole four-iron cluster.

Further indirect evidence of a magnetic state is obtained from the photoelectron spectra of the 2p electron. A study of transition-metal ions (Rosencwaig *et al.*, 1971) showed that multi-electron (or 'shake-up') satellites, observed as peaks at about 4 eV higher binding energy than the parent photoemission peak, may be excited when the 3d level is partially filled but are not observed when the electron spins are paired, e.g. for Cu^+ or Zn^{2+} . The spectrum of the Fe 2p electrons in a specimen of *Clostridium* ferredoxin is shown in Fig. 2(c). In addition to the spin-orbit splitting into $2p_{3/2}$ and $2p_{1/2}$ lines, satellite lines at higher binding energies are clearly observed on both lines. These are taken to be multi-electron excitation satellites and indirectly and independently also suggest that the iron atoms are magnetic.

(ii) Reduced high-potential iron-sulphur protein

The high potential iron-sulphur protein from *Chromatium* contains four iron atoms per molecule and has a molecular weight of 10000, so it has less

iron than in *Clostridium* ferredoxin by a factor of about three. It was not possible to observe the iron 3s lines in this protein, but the stronger 2p lines were seen and their energies are given in Table 1. The

Fig. 2. Photoelectron spectra in the Fe 2p regions for (a) oxidized spinach ferredoxin, (b) reduced *Chromatium* high-potential iron-sulphur protein and (c) oxidized *Clostridium* ferredoxin

Spectra were recorded as described in the Experimental section.

spectrum is shown in Fig. 2(b) and satellites are seen at similar energies to those in *Clostridium* ferredoxin.

(iii) Oxidized spinach ferredoxin

Spinach ferredoxin contains only two iron atoms in a molecule of mol.wt. 10500, and the 3s photoelectron lines could not be observed. The 2p spectra were observed, however, and showed shake-up satellites as shown in Fig. 2(a). The energies of the observed lines are given in Table 1.

There is no evidence for differences in valence states between iron atoms in oxidized spinach ferredoxin and in reduced high-potential iron-sulphur protein, although at the present level of resolution, small differences cannot be ruled out.

Discussion

The similarity between the shake-up satellites in the 2p spectra of the three proteins suggests that the iron atoms are magnetic in high-potential iron-sulphur protein and spinach ferredoxin as well as in *Clostridium* ferredoxin.

The major problem of the photoelectron spectroscopy technique when applied to biological materials arises from the possibility of chemical change of the sample. Freeze-drying of the sample does not substantially affect the physical integrity of the protein, as indicated by the optical absorption and e.p.r. spectra of redissolved samples. However, photoelectron spectroscopy examines only a very thin (less than 5 nm) surface layer of the sample, and it is less easy to determine whether this is affected by the X-ray beam. This may be detected from the change in binding energy which may decrease by as much as 2 eV for a unit reduction of the charge on an ion. For example, several compounds of Cu^{2+} have been found to be reduced by X-rays in a photoelectron spectrometer (Wallbank *et al.*, 1974), although there is no evidence so far for reduction on such a large scale occurring for an iron compound. In the present work, chemical shifts suggest that no reduction has occurred. High-potential iron-sulphur protein has a positive redox potential (+350 mV compared with -390 mV for *Clostridium* ferredoxin) and, as studied, is in its normal reduced state, and so is less likely to become further reduced by the X-rays. Since the 2p

spectra for oxidized *Clostridium* ferredoxin and reduced *Chromatium* high-potential iron-sulphur protein are very similar (Fig. 2) it is probable that the ferredoxin has not been reduced in the spectrometer, and the results that we have obtained therefore throw light on the state of the iron in the biologically active state.

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