

# Electronic state of iron in hemoglobin, myoglobin, and derivatives, as inferred from x-ray fluorescence spectra

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## Electronic State of Iron in Hemoglobin, Myoglobin, and Derivatives, as Inferred from X-Ray Fluorescence Spectra

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The Fe  $K\beta$  fluorescence emission spectrum was used to study the coordination of iron in some heme proteins. The spectrum was found to be dependent only on the direct environment of the iron. The iron atom in oxyhemoglobin can be regarded as trivalent, with a considerable negative charge on the outer oxygen atom of the  $O_2$  ligand. Carbonmonoxide hemoglobin contains divalent, zero spin iron. Methemoglobin is a low spin compound.

### INTRODUCTION

The way in which the iron atom is liganded in the heme proteins and related compounds has been the subject of considerable effort, both experimental and theoretical. The main experimental techniques thus far have been measurement of the magnetic moment,<sup>1</sup> visible and ultraviolet absorption spectrometry,<sup>2</sup> and, in recent years, Mössbauer spectrometry<sup>3</sup> and electron paramagnetic resonance.<sup>4</sup> Each of these techniques sheds a different light on the complex unity which a heme protein is, and gradually the role of the iron in the substances has become more clear. However, there still exist inconsistencies. The present study was undertaken to provide another tool for probing the the iron atom. It is well known that an x-ray fluorescence emission spectrum is influenced by the chemical bond in which the emitting atom takes part. For the  $3d$  transition metals, for instance, the relation was shown<sup>5</sup> between shifts and satellites of the main peaks of the K spectrum and the nature of the chemical bond. Iron proved to be an especially suitable element; it was even possible<sup>6</sup> to deduce its valence and coordination within reasonable limits in a class of inorganic compounds. Hence, this method was tried to probe the iron in heme proteins.

### USE OF THE FE $K\beta$ X-RAY SPECTRUM

The Fe  $K\beta$  spectrum consists of two main lines,  $K\beta_{1,3}$  which corresponds to a transition between the  $M_{II,III}$  level and the K level of the iron atom, and  $K\beta_5$ , much weaker, a transition between the  $M_{IV,V}$  level and the K level. Furthermore, a number of satellites may appear. Information may be drawn from shifts of the main lines ( $K\beta_5$  shifting is more

or less proportional to the charge on the iron atom) and the appearance of the other lines. Since the intensities obtainable for the proteins are low, especially of  $K\beta_5$ , shifts are not likely to be measurable with any precision. The only feature of the spectrum which can be measured rather reliably is the  $K\beta'$  peak. Fortunately, this peak is highly relevant for the present purpose.

Although the existence of  $K\beta'$  (to the low energy side of  $K\beta_{1,3}$ ) has been known since the 'twenties, its theoretical explanation is still not settled satisfactorily. A review of the theories brought forward has been given by Ekstig *et al.*<sup>7</sup>; these authors attribute the formation of  $K\beta'$  to the interaction between a hole in the  $3p$  shell and the incomplete  $3d$  shell, causing splitting of the  $M_{II,III}$  level. The agreement between calculated and observed spectra is still rather poor, however. Koster and Mendel<sup>5</sup> favor the view that part of the  $K\beta_{1,3}$  energy is consumed in promoting an unpaired  $3d$  electron into an excited state (into the conduction band); consequently a low energy satellite of  $K\beta_{1,3}$  should appear. This theory requires that the relative intensity of  $K\beta'$  for a given element should be proportional to the number of unpaired  $3d$  electrons present in the compound. Experiments on a large number of compounds of the elements titanium to nickel<sup>5,8</sup> indicate this to be the case within good approximation. Figure 1 may serve as an illustration. Iron(III) oxide, which contains the  $3d^5$  configuration with five parallel spins has a well-pronounced  $K\beta'$  satellite. In  $K_4Fe(CN)_6$ , where the six valence electrons of  $Fe^{2+}$  are engaged in  $d^2sp^3$  hybridization, no  $K\beta'$  peak can be seen; identical Fe  $K\beta_{1,3}$  profiles occur in for instance  $FeS_2$  and  $Fe(CO)_5$ , where the same situation prevails. Such a profile can be used

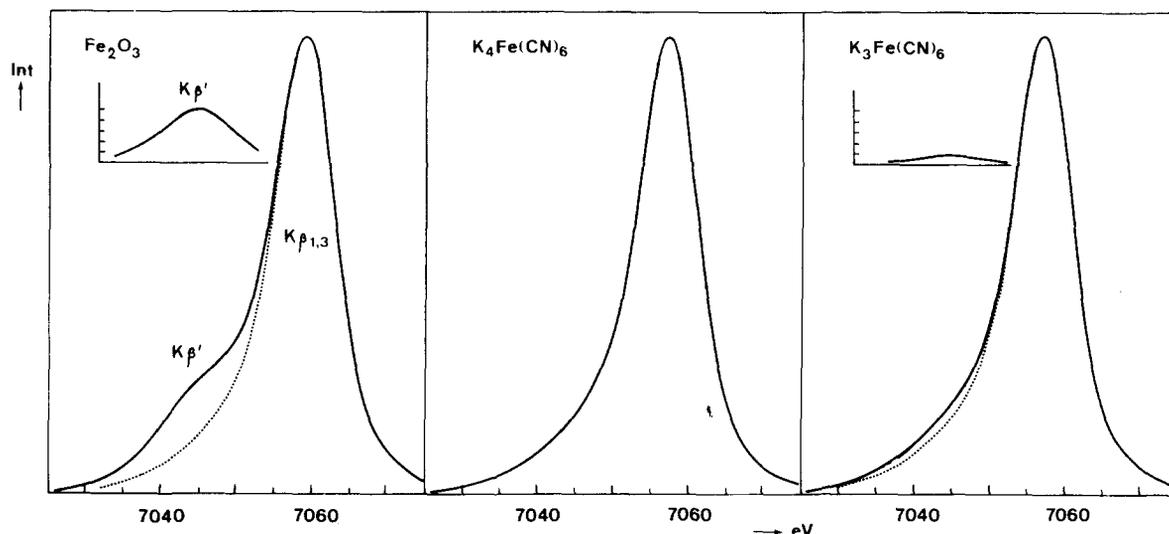


FIG. 1. The Fe  $K\beta_{1,3}\beta'$  spectra of three iron compounds. The horizontal axis gives the energy in electron volts, the vertical axis the normalized intensity. The dashed line represents the reference spectrum of  $K_4Fe(CN)_6$ .

as a reference profile. The Fe  $K\beta_{1,3}\beta'$  profile of  $K_3Fe(CN)_6$  does show a small  $K\beta'$  peak, in accordance with the notion that there is one unpaired 3d electron. The relative intensities of  $K\beta'$  in  $Fe_2O_3$  and  $K_3Fe(CN)_6$  have the ratio 5:1 in good approximation. So we may hope to apply this peak for the heme proteins as well. Hemoglobin and myoglobin were selected, while hemin [iron(III) protoporphyrin chloride] was also studied.

### EXPERIMENTAL

Two limiting factors are encountered when recording the x-ray spectra. First, heme proteins emit only a weak Fe  $K\beta$  fluorescence radiation, since they contain only a small amount of iron. This difficulty can normally be overcome by choosing long counting times, but proteins are rather sensitive to the white x-ray irradiation which is used in x-ray fluorescence equipment, so large numbers of counts cannot be accumulated. Secondly, solid samples must be studied, since aqueous solutions decompose very rapidly when subjected to x-ray excitation.

Commercially available hemoglobins (bovine and horse) were used. They were dissolved in phosphate buffer  $pH=7.0$  to make a 5% solution, which was centrifugated.

To prepare desoxyhemoglobin (Hb), the solution was treated with a 20-fold excess of  $Na_2S_2O_4$  during 20 min and passed through a Sephadex G25 column. The salt-free solution was dried in a desiccator under a nitrogen atmosphere over  $P_2O_5$ ; when most of the water was removed, a trace of  $Na_2S_2O_4$  was added to ensure complete reduction. As soon as the sample was dry, it was transferred into an x-ray fluorescence sample holder with mylar window, a glove box being used to protect the sample from atmospheric oxygen.

To prepare oxyhemoglobin ( $HbO_2$ ), the salt-free solution, obtained as described above, was saturated with oxygen gas during 20 min. Then the solution was concentrated to dryness in a desiccator under an oxygen atmosphere over  $P_2O_5$ .

Carbonmonoxidehemoglobin ( $HbCO$ ) was prepared in the same manner using carbon monoxide gas.

To prepare methemoglobin (metHb), the buffered solution was treated with a 20-fold excess of  $NaNO_2$  during 60 min and passed through a Sephadex G25 column. The solution was evaporated in vacuum (1 cm Hg) over  $P_2O_5$ .

The solid samples obtained were completely soluble in buffer  $pH=7.0$ , and the optical absorption spectra of their solutions were checked. No deterioration of the proteins could be detected during this drying process, except for  $HbO_2$ , which invariably contained 10%–25% of methemoglobin. Only those samples which contained at most 10% metHb were investigated with the x-ray spectrometer.

Freeze drying was also employed to concentrate the protein solutions. The x-ray spectra were similar, but less intense and hence less accurate, because the density of the freeze-dried powders is much less than the density of powders produced as described above.

A commercially available myoglobin preparation (horse) was used in the same way to prepare metmyoglobin (metMb).

The spectra were recorded on a 2 kW Philips semi-automatic spectrometer PW 1220, using a Mo primary target at 40 kV/40 mA and a LiF analyzing crystal in the second order. A measurement was performed at steps of  $0.1^\circ$  in  $2\theta$ , between  $121.6^\circ$ – $122.6^\circ$   $2\theta$ . Care was taken that each sample was subjected to x-ray excitation for at most 20 min, and its optical spectrum was checked after the irradiation. At least three

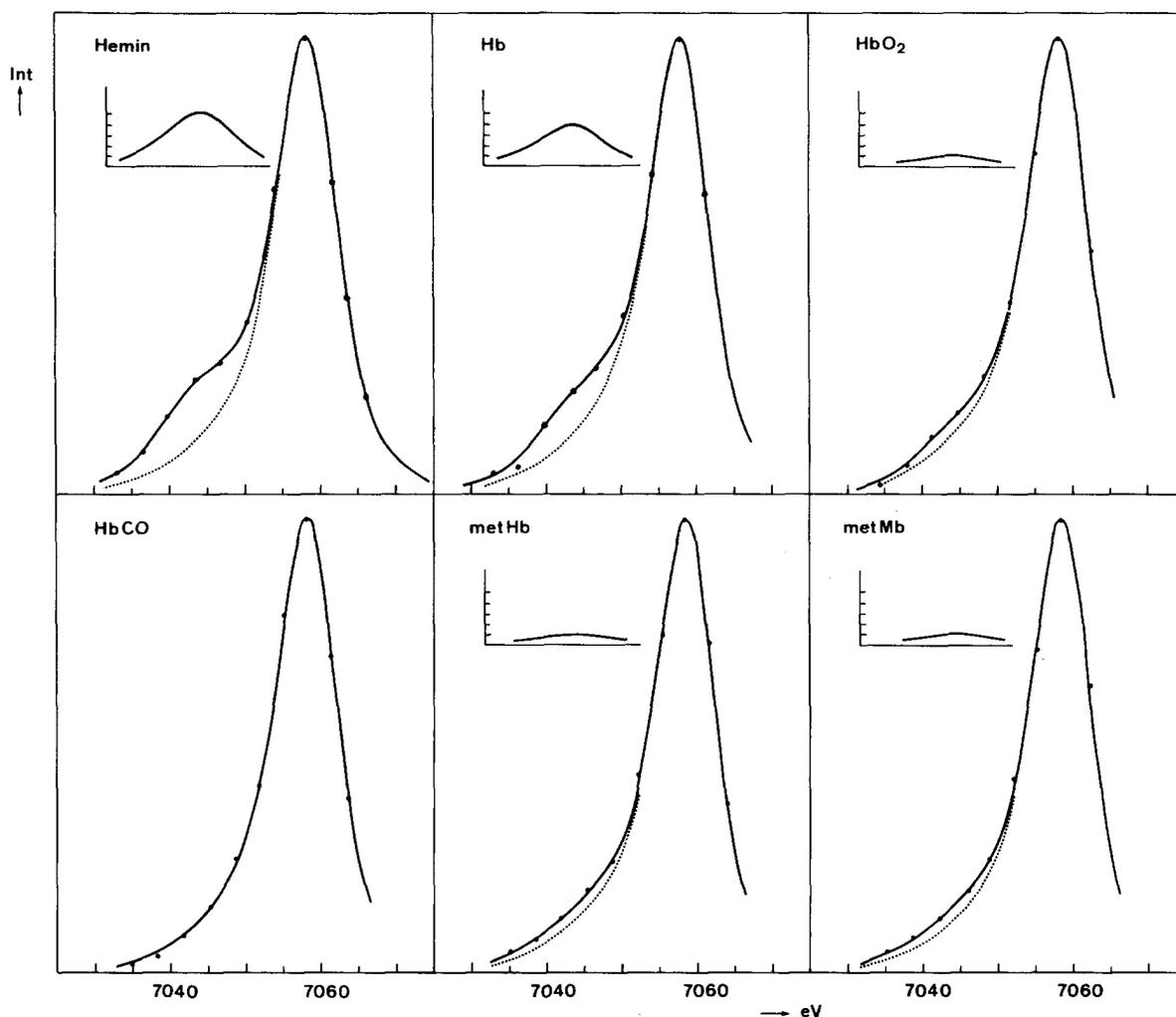


FIG. 2. The Fe  $K\beta_{1,3}\beta'$  spectra of hemin, bovine Hb,  $HbO_2$ , HbCO, and metHb and horse metMb. The count rate at peak intensity was 6000–9000 counts/400 sec, using molybdenum excitation at 40 kV/40 mA.

samples were prepared of each substance, and at least three runs were made of each batch.

### RESULTS AND CONCLUSIONS

Representative spectra are shown in Fig. 2. It is apparent that the method essentially probes the iron atom and its immediate neighbors; a sample of a bovine hemoglobin derivative and one of equine origin give the same spectrum, likewise the metMb spectrum is identical with the metHb spectrum. In fact, this rule is found to be obeyed in almost all investigations of x-ray spectra, those bonds only exerting influence which have a considerable energy. The environment of the heme iron can for the present purpose be described as consisting of the four pyrrole groups of the nearly plane heme group, an imidazole nitrogen atom of a proximal histidine somewhere in the globine chain and (except probably in Hb) a sixth ligand on the other side of the heme group.

The Fe  $K\beta_{1,3}\beta'$  spectrum of hemin closely resembles

that of  $Fe_2O_3$ ; hemin contains an  $Fe^{3+}$  ion with five parallel spins. This is to be expected, the  $Fe^{3+}$  ion not being able to hybridize into a covalent planar square coordination. In fact the Fe lies considerably out of the plane of the four nitrogen atoms (Koenig<sup>9</sup>).

The spectrum of Hb shows that the  $Fe^{2+}$  ion contains four parallel spins; this was already found by Pauling and Coryell<sup>1</sup> in their classical work on the magnetic moments of hemoglobin derivatives. These magnetic moments were determined in solution; we find the same number of parallel spins in a solid sample, which shows that our way of drying (rather rapidly, and not prolonged after the sample became solid) yields a product whose molecules are comparable to the dissolved molecules as regards the heme environment. If Hb is dried for many hours, a dehydrated molecule is formed, as is witnessed by spectral data of Trautwein, Eicher and Mayer.<sup>10</sup>

The magnetic moment of  $HbO_2$  is zero,<sup>1</sup> and it is assumed that by combining Hb with paramagnetic

oxygen all spins become paired. The x-ray spectrum shows, however, that the iron atom still possesses one unpaired electron. This contradiction can be explained by recalling that the x-ray fluorescence analysis probes only the iron atom and its immediate vicinity, whereas the magnetic susceptibility is determined by the magnetic moment of the molecule as a whole. The unpaired electron in HbO<sub>2</sub> can only be explained by assuming that the iron is octahedrally coordinated by means of  $d^2sp^3$  hybridization, as in the complex cyanides, and that the iron is actually trivalent. Weiss<sup>11</sup> pointed out already that this might be the case, and since then experimental evidence in favour of this concept has been given by Maling and Weissbluth,<sup>3</sup> using Mössbauer spectroscopy, although Eicher and Trautwein<sup>4</sup> with the same technique came to another viewpoint, and by Wittenberg *et al.*,<sup>12</sup> who combined optical absorption and EPR data. A formal charge of 3+ on the iron means that the O<sub>2</sub> ligand is effectively the O<sub>2</sub><sup>-</sup> ion. The outer oxygen atom of this ion may be supposed to carry most of the negative charge, in accordance with a model of Pauling<sup>13</sup>; in such a way electrostatic bonding occurs with the positively charged imidazole nitrogen atom of the distal histidine. The O<sub>2</sub><sup>-</sup> ion is known to possess one spin,<sup>14</sup> which combines with the one spin of the iron atom to yield diamagnetic HbO<sub>2</sub>. By assuming that the iron atom is essentially trivalent, it is readily understood why this substance is relatively stable to oxidation: it contains already Fe<sup>3+</sup>.

The spectrum of HbCO shows that no unpaired 3d electrons are present; it can be described as an iron(II) compound in which the two bonding electrons of the CO molecule are utilized to form an

octahedral complex with  $d^2sp^3$  hybridization. The bonds in HbO<sub>2</sub> and HbCO are thus dissimilar, which seems to follow also from Mössbauer spectra.<sup>3,4</sup>

The spectra of metHb and metMb indicate that the iron atom is trivalent, with one unpaired electron and six ligands in  $d^2sp^3$  hybridization. They are evidently "low spin" compounds. This is in contradiction to measurements of the magnetic moment, which is consistent with a "high spin" structure of both hydrate and hydroxide of metHb. EPR spectroscopy, however, has shown the hydroxide to be "low spin"<sup>12</sup>; probably a difference in the degree of hydration has brought about these inconsistencies.

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