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The State of Iron in Hemerythrin. A Mössbauer Study*

M. Y. Okamura, I. M. Klotz, C. E. Johnson, M. R. C. Winter, and R. J. P. Williams

ABSTRACT: Mössbauer spectra, and some ancillary magnetic susceptibilities, have been measured for hemerythrin in its deoxy, oxy, and several met states, as well as for a series of model binuclear, oxygen-bridged, iron compounds. Isomer shifts and quadrupole splitting values for iron in the protein derivatives are comparable to those seen in the antiferromagnetically coupled iron model compounds. The Mössbauer spectra together with the susceptibility data and the known chemical nature of the methemerythrin complexes indicate that the two iron atoms in hemerythrin are in similar environments and are near enough to interact with each other.

The data also provide evidence that the bridge formulation, FeO_2Fe, for the active site of hemerythrin is correct.

Hemerythrin is the non-heme iron protein responsible for oxygen transport in representatives of four different invertebrate phyla (Ghiretti, 1962). Neither the binding of the iron to the protein, nor the binding of oxygen to the iron is well understood. In an attempt to improve our knowledge of these phenomena, we have studied the magnetic susceptibility and the Mössbauer spectra of hemerythrin.

Hemerythrin has a molecular weight of 108,000 and is composed of eight subunits (Klotz and Keresztes-Nagy, 1963) each of which contains two iron atoms and can bind 1 mole of oxygen. Since it is likely that the two iron atoms in each subunit are involved in oxygen binding, and are hence close together, the Mössbauer spectra should be easier to interpret than optical or magnetic resonance properties of the protein. In particular, it has been found recently (Johnson, 1966b) that the effects of strong magnetic fields on Mössbauer spectra provide criteria for characterizing iron in biological compounds. These criteria can be applied to hemerythrin also to determine whether the iron atoms are interacting magnetically and hence in close proximity.

The oxygen is believed to be bound in a bridging manner between the two iron atoms in a similar way to the binding in synthetic cobalt(II) binuclear oxygen carriers (Vogt *et al.*, 1963). The iron atoms have been assumed to be in the iron(II) state in deoxyhemerythrin, but in oxyhemerythrin the valence state could be formulated in a number of different ways, *e.g.*, Fe(III)-O_2^{2-}-Fe(III) (Klotz and Klotz, 1955) or Fe(II)-O_2-Fe(II) (Williams, 1955). In all these cases the iron could be in either high- or low-spin states.

The iron in the deoxy and oxy species can be oxidized to the iron(III) species, which can bind a variety of ligands (Keresztes-Nagy and Klotz, 1965) and could also be in either a high- or low-spin state.

A further complication arises in the iron(III) forms and in the high spin iron(II) form if, as may well be the case, the two iron atoms are binding one bridging ligand. (It has been demonstrated that in the case of azide and thiocyanate the ligands are bound in the ratio of 1 mole of ligand:2 moles of iron; Keresztes-Nagy and Klotz, 1965; Klapper and Klotz, 1968.) The iron–ligand–iron system could now give rise either to weak (antiferromagnetic) or strong (bonded) interactions.

Many of the above possibilities are open to investigation by Mössbauer spectroscopy, but in order to be confident of the interpretation, it was necessary to prepare and study simple compounds having iron–iron interactions. The compounds which we have prepared have been described previously (Lewis *et al.*,

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TABLE 1: Analytical Data for Model Complexes.

Compound	Calcd (%)			Found (%)		
	C	H	N	C	H	N
$(Fe(salen)Cl)_2$	53.7	4.0	7.8	53.22	3.87	7.70
$[(Fe(phen_2)Cl)_2O]Cl_2 \cdot 6H_2O$	52.5	4.0	10.2	53.09	3.82	10.45
$(Fe(salen))_2O$	58.22	4.28	8.49	58.01	4.38	8.40
$enH_2[(Fe(HEDTA))_2O] \cdot 6H_2O$	31.15	6.18	9.91	31.15	6.27	9.80

1967; Khedekar *et al.*, 1967; Gerloch *et al.*, 1968; Schugar *et al.*, 1967) and are listed in Table I. They are all binuclear species, formally derivatives of iron(III) and fall into two classes.[1] $(Fe(salen)Cl)_2$ is known from X-ray single crystal analysis (Gerloch and Mabbs, 1967b) to involve hexacoordinated iron atoms and there are two bridging oxygen atoms each derived from different ligand molecules. The compound $[(Fe(phen)_2Cl)_2O]Cl_2 \cdot 6H_2O$ is also believed to be hexacoordinate. $(Fe(salen))_2O$, however, is believed to be a five-coordinate species with a bridging oxygen between the two metal atoms. Magnetic data (Lewis *et al.*, 1967; Khedekar *et al.*, 1967; Gerloch *et al.*, 1968) indicate that these dimeric compounds are high-spin antiferromagnetically coupled iron(III) systems.

We have also prepared a mixture of the pentacoordinate monomer (Gerloch and Mabbs, 1967a) and hexacoordinate dimer of (Fe(salen)Cl) in order to compare the Mössbauer spectra from antiferromagnetically coupled iron atoms with those from a closely related noninteracting system.

We describe below the Mössbauer spectra of these derivatives together with preliminary data on the binuclear O-bridged compound $enH_2[(FeHEDTA)_2O]$. A much more detailed study of the Mössbauer spectra of the binuclear model compounds at 300 and 77°K has been undertaken by Bennett *et al.* (1968) and we shall not publish our detailed work at these temperatures since we agree in all respects with their results.

Experimental Section

Model Compounds. The salen and *o*-phen derivatives and $enH_2[FeHEDTA]_2O \cdot 6H_2O$ were prepared and recrystallized exactly as described in the literature (Lewis *et al.*, 1967; Khedekar *et al.*, 1967; Gerloch *et al.*, 1968; Schugar *et al.*, 1967). Analytical data for these derivatives are given in Table I. The monomer dimer mixture of (Fe(salen)Cl) was prepared by recrystallization of the pure dimer from nitromethane (Gerloch and Mabbs, 1967a). Analytical results for this material were not obtained because the ratio of solvent-containing monomer crystals to solvent-free dimer crystals is not known. The $enH_2[FeHEDTA]_2O$ compound was recrystallized from dimethylformamide and washed with alcohol.

Preparation of Hemerythrin. Oxyhemerythrin was prepared as previously described (Klotz *et al.*, 1957) and crystallized from a solution of 20% alcohol containing 0.4% NaCl.

Metaquohemerythrin was prepared by oxidizing a concentrated solution ($1–2 \times 10^{-2}$ M in Fe) of oxyhemerythrin dissolved in 0.1 N Tris-cacodylate (pH 8.5) buffer using a 1.2–1.5-fold excess of potassium ferricyanide. The excess ferricyanide and ferrocyanide were removed by passage of the solution through an anion-exchange column in the perchlorate form, and the effluent was dialyzed extensively against 0.1 N Tris-cacodylate (pH 8.5) buffer to remove bound chloride. The solution was then dialyzed against 0.1 N $NaClO_4$. Crystals of metaquohemerythrin form as the solution stands at 4°.

Methemerythrin thiocyanate crystals were prepared from a concentrated solution of metaquohemerythrin to which enough solid potassium thiocyanate was added to make a 0.1 M SCN^- solution. Methemerythrin thiocyanate crystallized after dialysis of the solution against distilled water.

Deoxyhemerythrin crystals were prepared by dissolving oxyhemerythrin in 0.1 M Tris-cacodylate buffer (pH 8.5) and then dialyzing the solution against 0.1 M Tris-cacodylate buffer (pH 7.3) containing 0.1 M D-glucose. The solution was then transferred to a specially designed polyethylene bag. Glucose oxidase (Sigma, 200 units) containing a trace of catalase (~0.1 mg) was added to the solution and the top of the bag was pinched off. Needles of deoxyhemerythrin formed from the slightly yellow solution and settled into the pouch-shaped lower portion of the bag. The pouch was then pinched off and heat sealed.

Mössbauer Spectroscopy. Mössbauer spectra were measured using the spectrometer designed by Cranshaw (1964). Spectra were calibrated using the known positions of the peaks of the spectrum of metallic iron (Preston *et al.*, 1962) and taking the center of symmetry of this spectrum as the velocity zero.

Spectra were measured at 4.2, 77, and 195°K in the absence of an externally applied magnetic field, and at 4.2°K in the presence of such a field. Results were plotted using a data-plotting routine designed for Mössbauer spectroscopy by M. Ridout (personal

[1] Abbreviations used: salen, *N,N'*-bissalicylideneethylenediamine; en, ethylenediamine; HEDTA, *N*-hydroxyethylethylenediaminetriacetate; phen, 1,10-phenanthroline; acac, acetylacetonate; py, pyridine.

communication, 1968). This routine plots the percentage transmission as a function of source velocity (relative to iron metal).

Electron Paramagnetic Resonance Measurements. Electron paramagnetic resonance studies were carried out primarily with oxyhemerythrin. In particular signal intensities were followed as the protein was titrated with ferricyanide.

Oxyhemerythrin crystals were dissolved in 0.1 M Tris-cacodylate buffer (pH 8.5) and the solution was divided among four test tubes. The iron concentration was 2.8×10^{-3} M as determined by the absorption at 500 mμ. Varying amounts of $K_3Fe(CN)_6$ were added to each tube and reaction was allowed to proceed overnight at 4°. The optical spectra were taken after dilution of each sample to a convenient concentration. The electron paramagnetic resonance spectra were obtained, on the undiluted sample, on a Varian EP-3 spectrometer at 77°K in a liquid nitrogen dewar flask.

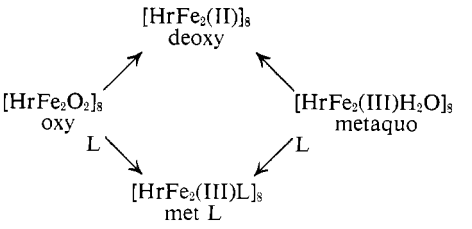
Magnetic susceptibility measurements were made using the shape-factor nuclear magnetic resonance method of Mulay and Haverbush (1964), on the Varian A-60 nuclear magnetic resonance spectrometer. The separation of the two peaks of the reference liquid, bromoform, which arises from the spherical and cylindrical portions of the reference capillary, is linearly related to the volume susceptibility of the sample, as verified by calibration with organic liquids and nickel(II) chloride solutions. The constant of proportionality was 2.02 ± 0.02 (ppm/cgs units × 10^6). Measurements were made at probe temperature and also at lower temperatures using the Varian regulator. Temperature measurements were made using the splitting in a standard methanol sample.

Differences between the magnetic susceptibility of various derivatives were measured in order to circumvent the problem of determining the diamagnetic contribution of the protein and buffer. Measurements were made on the initial protein solution and the product prepared by the addition of a small volume of reactant directly into the nuclear magnetic resonance tube. The optical spectra of the initial solutions and products were obtained with a Cary 14 spectrophotometer and were used to determine the iron concentrations (Keresztes-Nagy and Klotz, 1965).

The deoxygenation of oxyhemerythrin was accomplished directly in the nuclear magnetic resonance tube by addition of 0.05 ml of glucose oxidase (50 units) to 0.80 ml of oxyhemerythrin in 0.1 M D-glucose. The same amount of buffer was added to the initial protein solution to correct for dilution. Deoxyhemerythrin was also prepared by addition of solid sodium dithionite to oxyhemerythrin or to metaquohemerythrin. Various liganded met derivatives were prepared by addition of small volumes of a concentrated solution of the ligand to either metaquohemerythrin or to oxyhemerythrin.

Results and Discussion

Magnetic Susceptibility. Changes in magnetic susceptibility were measured for the conversions of hemerythrin among the following forms



where L represents added ligand.

No appreciable changes in susceptibility occurred when oxyhemerythrin was converted into the met-azide or met-fluoride form, or when met-aquohemerythrin was changed to the azide, cyanide or thiocyanate complex. However, the magnetic susceptibility increased when deoxyhemerythrin was prepared from either oxyhemerythrin or metaquohemerythrin (Table II).

The magnetic susceptibility data show that oxyhemerythrin and the derivatives of methemerythrin exhibit a lower effective magnetic moment than does deoxyhemerythrin, which contains iron(II). The low effective magnetic moment in the iron(III) compounds could in principle be ascribed either to spin pairing due to a strong ligand field or to an antiferromagnetic interaction between the pair of iron atoms in each protein subunit. The Mössbauer results support the latter interpretation.

The difference in susceptibility between deoxyhemerythrin and oxyhemerythrin is slightly lower than that found in high-spin iron(II) compounds (Figgis and Lewis, 1964) and may be due either to a small magnetic moment in oxyhemerythrin or to some antiferromagnetic interaction between iron atoms in deoxyhemerythrin.

Electron paramagnetic resonance spectra of oxyhemerythrin have been reported previously (Beinert *et al.*, 1962). The spectrum consists of an absorption at $g = 2.00$ which is reduced in intensity when the protein is deoxygenated. However the signal can be associated with only a small portion of the total iron in the sample (H. Beinert, private communication). Our results from the electron paramagnetic resonance titration definitely establish that the $g = 2$ signal is not correlated with the optical spectrum. In fact the electron paramagnetic resonance signal is eliminated upon addition of only a fraction of the oxidant required for titration of all the iron (Table III).

The signal at $g = 2$ could be a free radical produced by Fe(III) oxidation of the protein. This would account for the reduction of the intensity upon deoxygenation. The absence of an easily observable electron paramagnetic resonance signal due to iron is not surprising since the same processes which eliminate magnetic hyperfine interaction in the Mössbauer spectra described below also tend to broaden the electron paramagnetic resonance spectra.

Signals at $g = 2$ have also been observed with methemerythrins. These cannot account for more than 10% of the iron present.

TABLE II: Changes in Magnetic Susceptibility on Conversion of Hemerythrin from One Form into Another.

Initial State	Final State	Substances Added[a] for Conversion	Temp (°K)	$\Delta\chi \times 10^6$/ Mole of Fe
$[HrFe_2O_2]_8$	$[HrFe_2(II)]_8$	Glucose oxidase, 0.04 ml (10 mg/ml)	297	7500
$[HrFe_2O_2]_8$	$[HrFe_2(II)]_8$	Glucose oxidase, 0.05 ml	304	6900
$[HrFe_2O_2]_8$	$[HrFe_2(II)]_8$	Sodium dithionite, solid	304	7600
$[HrFe_2(III)H_2O]_8$	$[HrFe_2(II)]_8$	Sodium dithionite, solid	304	7400
$[HrFe_2O_2]_8$	$[HrFe_2N_3]_8$	NaN_3, 0.04 ml (5 M)	297	700[b]
$[HrFe_2O_2]_8$	$[HrFe_2F]_8$	KF, 0.05 ml (2 M)	304	700[b]
$[HrFe_2(III)H_2O]_8$	$[HrFe_2(III)N_3]$	NaN_3, 0.01 ml (5 M)	304	−400[b]
$[HrFe_2(III)H_2O]_8$	$[HrFe_2(III)CN]$	NaCN, 0.04 ml (0.02 M)	304	−900[b]
$[HrFe_2(III)H_2O]_8$	$[HrFe_2(III)SCN]_8$	KSCN, 0.02 ml (5 M)	304	±500[b]

[a] In each case addition was to 0.80 ml of solution in a nuclear magnetic resonance tube. [b] Experimentally these are not significantly different from zero.

Mössbauer Spectra. Isomer shift, δ, and quadrupole splitting, Δ, data for the compounds studied are reported in Table IV, and representative spectra are shown in Figures 1–8. Comments upon the data will be given in the following order: (a) iron(II) hemerythrin, (b) iron(III) models, (c) iron(III) hemerythrin, and (d) oxygenated hemerythrin. In this way the most relevant comparison between models and hemerythrin oxidation state is made.

IRON(II) COMPOUNDS. Deoxyhemerythrin (Figure 1) shows a simple quadrupole split pair of lines at all temperatures in the absence of a magnetic field, indicating that, within the limits of our data, there is but one iron environment. (The small peak at about +1.5 mm sec^{-1} in this sample is probably due to a trace of iron(III) impurity.) The relatively high value of the isomer shift, δ, 1.19 mm sec^{-1}, and the large quadrupole splitting, Δ, 2.81 mm sec^{-1} show the compound to be high-spin iron(II). In comparison, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ gives δ = 1.40 mm sec^{-1} and Δ = 2.70 mm sec^{-1} at 77°K (Brady *et al.*, 1965). The lower value of δ in hemerythrin suggests that nitrogen as well as oxygen ligands could be involved in binding. Spectra were not obtained of this compound in a magnetic field due to instrumental difficulties when this sample was being studied.

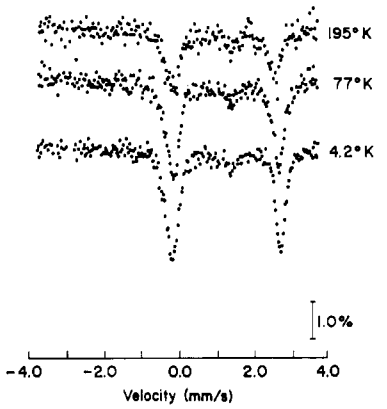


FIGURE 1: Mössbauer spectra of deoxyhemerythrin, at temperatures indicated.

IRON(III) COMPOUNDS. Before considering the oxygenated derivative and the ferric derivatives of the protein, we shall mention the simpler model binuclear complexes whose magnetic properties are fairly well understood. $(Fe(salen)Cl)_2$, $(Fe(salen))_2O$, and $[(Fe(phen_2)Cl)_2O]Cl_2 \cdot 6H_2O$ which have diamagnetic ground states, give quadrupole split spectra having both lines of equal intensity at 4.2°K (*e.g.*, Figure 5). Application of a 5-kgauss magnetic field causes little broadening of the spectrum. $(Fe(salen)Cl)_2$ and $(Fe(salen))_2O$ were

TABLE III: Titration of Optical Absorbance and Electron Paramagnetic Resonance Signal of Oxyhemerythrin with $K_3Fe(CN)_6$.

Tube[a]	$K_3[Fe(CN)_6]$ Added (moles/l.)	% Change in Absorbance at 500 mμ	% Change in Amplitude of Electron Paramagnetic Resonance Signal
1	0	0	0
2	0.001	33	98
3	0.002	59	100
4	0.005	100	100

[a] Fe concentration in undiluted oxyhemerythrin sample was 2.8×10^{-3} M.

TABLE IV: Zero-Field Isomer Shift and Quadrupole Splitting Data.

Compound	Temp (°K)	Δ, mm sec^{-1} (±0.05 mm sec^{-1})	δ, mm sec^{-1} (±0.05 mm sec^{-1})
$(HrFe(II)_2)_8$	195	2.75	1.11
	77	2.81	1.19
	4.2	2.89	1.20
$(HrFe(III)_2NCS)_8$	77	1.81	0.52
$(HrFe(III)_2OH_2)_8$	77	1.57	0.46
$(HrFe_2O_2)_8$			
Outer pair	77	1.93	0.51
	4.2	1.92	0.54
Inner pair	77	1.03	0.48
	4.2	1.09	0.51
$(Fe(salen)Cl)_2$	300	1.38	0.39
	77	1.39	0.48
	4.2	1.38	0.51
(Fe(salen)Cl)	300	1.32	0.37
Monomer + dimer	77		
	4.2	1.39	0.45
$[(Fe(phen_2)Cl)_2O]Cl_2 \cdot 6H_2O$	77	1.70	0.46
	4.2	1.50	0.47
$(Fe(salen))_2O$	77	0.84	0.41
	4.2	0.85	0.46
$enH_2[(Fe(HEDTA))_2O]$[a]	298	1.63	0.40

[a] Preliminary data obtained on different Mössbauer spectrometer.

studied in higher magnetic fields (Figures 6 and 7). The two peaks of the quadrupole split pair are further split, one into a doublet and the other into a triplet, in these large fields, confirming a diamagnetic system. The doublet is at low velocity in the chloride showing that the electric field gradient at the iron nucleus is negative in this compound. In the oxide derivative, however, the positions of the doublet and triplet are reversed showing the presence of a positive electric field gradient here. The quadrupole splitting is small, so that the hyperfine peaks arising from each zero field peak overlap at 30 kgauss. The spectrum at 25 kgauss shows the hyperfine splitting best. More work needs to be done on these systems to elucidate the factors governing the sign of the electric field gradient.

The isomer shift values are in the normal range for high-spin iron(III) compounds. The quadrupole splittings for the six-coordinate dimers, $(Fe(salen)Cl)_2$, $[(Fe(phen_2)Cl)_2O]Cl_2 \cdot 6H_2O$, and $enH_2[(Fe(HEDTA))_2O]$, are all near 1.5 mm sec^{-1} which is higher than most conventional high-spin iron(III) compounds (Δ = 0–1.0 mm sec^{-1}). The value for the five-coordinate $[(Fe(salen))_2O]$ dimer is much lower and at the high end of the range found for conventional high-spin iron(III) compounds.

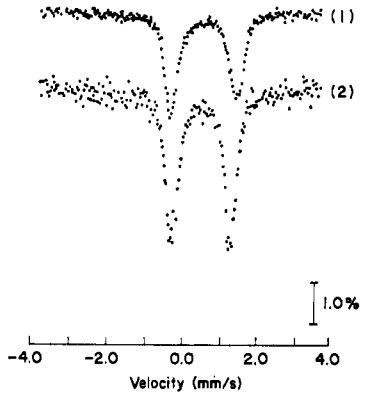


FIGURE 2: Mössbauer spectra of thiocyanatohemerythrin (1) and aquohemerythrin (2) at 77°K.

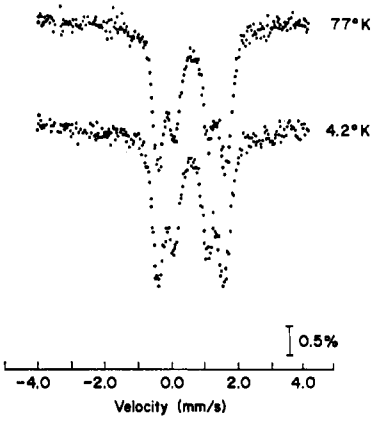


FIGURE 3: Mössbauer spectra of oxyhemerythrin at temperatures indicated.

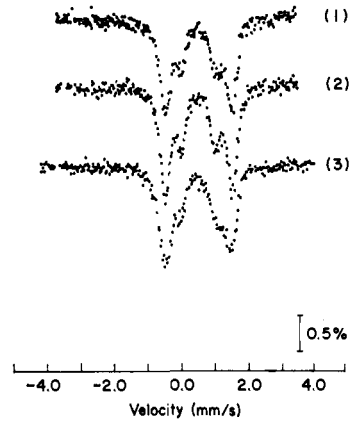


FIGURE 4: Mössbauer spectra of denatured oxyhemerythrin at 77°K; (1) kept at 277°K for 4 weeks, (2) thawed and frozen six times, and (3) kept at 300°K for 6 hr.

The paramagnetic monomer and diamagnetic dimer mixture of (Fe(salen)Cl) produced by recrystallization of the dimer from nitromethane gives a quadrupole splitting and isomer shift almost indistinguishable from those of the pure dimer (Figure 8). However, it differs from the dimer in two important ways. Although the dimer shows broadening of the high velocity line as the temperature is increased, this broadening is not very great at 77°K. The monomer–dimer mixture, however, shows quite appreciable broadening at this temperature. The second difference is in the behavior at 4.2°K in a magnetic field where the mixture shows behavior typical of a paramagnet. (The iron thus appears to be similar in behavior to that in hemin (Johnson, 1966a) except that the hyperfine spectrum is less symmetrical in (Fe(salen)Cl) indicating a great anisotropy of the hyperfine interaction in this compound.) Thus it appears that a bridged Fe–X–Fe system with no long-range ordering of spins can be readily recognized by study of the Mössbauer spectra obtained in a magnetic field, though little distinction

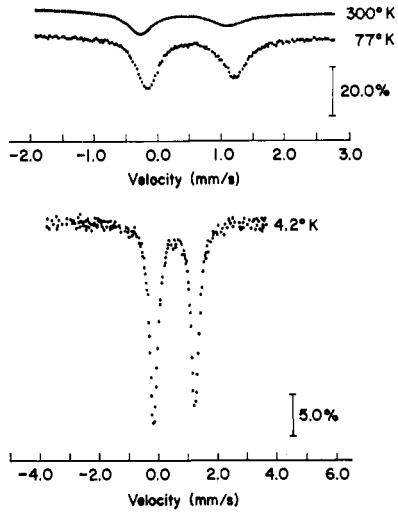


FIGURE 5: Mössbauer spectra of $(Fe(salen)Cl)_2$ at temperatures indicated.

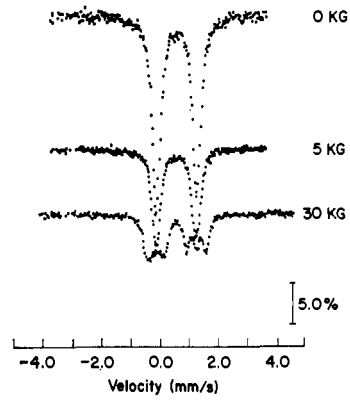


FIGURE 6: Mössbauer spectra of $(Fe(salen)Cl)_2$ at 4.2°K, in the presence of magnetic fields (in kilogauss) indicated.

may exist in either quadrupole splitting or isomer shift (Johnson, 1966b).

METHEMERYTHRINS. We shall now consider the iron(III) forms of the protein. These compounds have a lower magnetic susceptibility than deoxyhemerythrin (Table II). Iron(III) hemerythrin (Figure 2), both as the aquo and thiocyanato complexes, gives a simple quadrupole split spectrum indicating that here, as in the deoxy form, there is only one type of iron site in each of the compounds. Now the thiocyanate is formed from the aquo by the addition of *1* mole of SCN^-/*2* iron atoms. (The same is true of azide.) It is most likely, therefore, that the thiocyanate is acting as a bridging group, for addition of SCN^- to only one of the two irons should split the Mössbauer signal. These data together with the values of the isomer shifts (0.46 mm sec^{-1} for aquo- and 0.52 mm sec^{-1} for thiocyanatohemerythrin at 77°K) and the quadrupole splitting (1.57 and 1.81 mm sec^{-1}, respectively) which are very similar to the values for the high-spin binuclear iron(III) model compounds, strongly indicate that the simple quadrupole split spectrum of the Fe(III) hemerythrin compounds is that of a pair of coupled high-spin Fe(III) ions. The alternative proposition that the iron atoms are isolated (and that the observation of one simple quadrupole spectrum for the

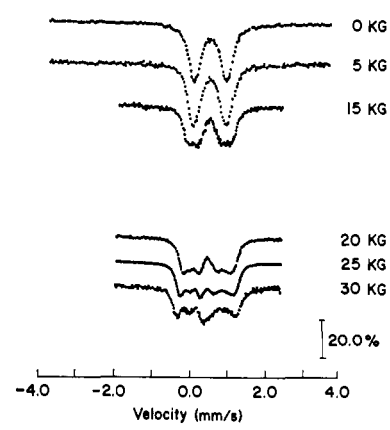


FIGURE 7: Mössbauer spectra of $(Fe(salen))_2O$ at 4.2°K, in the presence of magnetic fields (in kilogauss) indicated.

two atoms in both compounds is coincidental) can be eliminated by an examination of the Mössbauer spectrum in a magnetic field (Johnson, 1966b) (Figure 9). No magnetic hyperfine structure is seen in the signal.[2] This result also eliminates the possibility that the iron atoms are *two* isolated low-spin Fe(III) cations. A final possibility is that the iron(III) atoms could be low-spin-interacting cations, but this interpretation is most unlikely in view of the high isomer shift which is typical of that for high-spin cations and is higher than that of any known low-spin iron complex. *In toto* these studies (chemical, susceptibility, and Mössbauer spectroscopy) lead us to conclude that there is strong antiferromagnetic interaction between the iron atoms which are in high-spin state.

OXYGENATED COMPOUNDS. Oxyhemerythrin, which is of low susceptibility (compared with deoxyhemerythrin), gives rise to a spectrum consisting of two quadrupole split pairs of lines superimposed on each other in the absence of a magnetic field (Figure 3). The fact that the two outer lines increase in intensity while the two inner lines decrease with time (Figure 4) shows that the inner lines are certainly characteristic of the oxygenated species. The outer pair could arise from the conversion of the oxygenated species into the met form. Since it has proved to be impossible to prepare oxyhemerythrin entirely free from Fe(III) forms[3] (Klotz *et al.*, 1957), at least some of the intensity of the outer lines must arise from met forms. If the outer pair, however, also reflects the oxy form, then it is possible that a part of the intensity in this region is due to a peroxy-bridge structure Fe(III)–O_2^{2-}–Fe(III) which could have a Mössbauer spectrum very like that of the other methemerythrin derivatives just described.

At the low temperature of the Mössbauer measurements the oxy compound does not contain paramagnetic atoms, for a 5-kgauss magnetic field causes no broadening of the spectrum. This is in keeping with the low susceptibility at room temperature and with the absence of any electron paramagnetic resonance signal attributable to the iron in oxyhemerythrin. None of these data are at variance with the above explanations for the outer pair of lines, but they require that similar restrictions should be applied to the explanation of the inner pair of lines, which we stress must be assigned to a part, and possibly to the whole, of the iron of the true oxyhemerythrin. This inner pair of lines could arise from an alternative geometric arrangement of two bridged Fe(III) cations, for the values of $\delta(0.48)$ and $\Delta(1.03)$ are close to those for the binuclear complex $(Fe(salen))_2O$ (Table IV). However, the spectrum could also arise from a coupled spin system containing two Fe(II) atoms and an oxygen molecule. For example, $Fe(II)(acac)_2(py)_2$ gives $\Delta = 0.6$–0.8 mm sec^{-1}, and $\delta = 0.55$ mm sec^{-1} at room temperature (Epstein, 1962). Low-spin iron(III) would appear to be excluded since no compounds with this spin state having such a high value of δ are known.

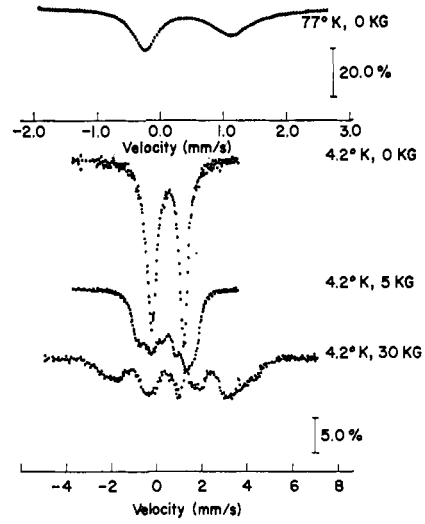


FIGURE 8: Mössbauer spectra of (Fe(salen)Cl) monomer plus dimer at temperatures and magnetic fields indicated.

Conclusion

In the absence of a method which definitely measures the spin density on the iron atoms we are not able to resolve the problem of the "oxidation state" of the iron. It could well be that this is an artificial problem and that we should discuss the oxidation state of the unit FeO_2Fe only.

The stoichiometry of the chemical reactions, the susceptibility measurements, and the Mössbauer spectra in the presence and absence of a magnetic field

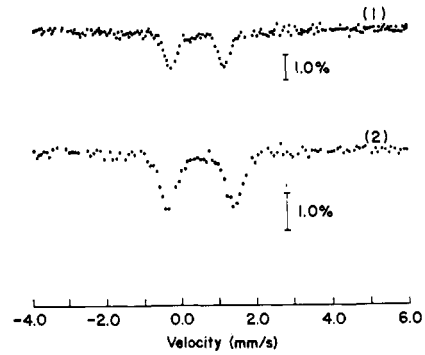


FIGURE 9: Mössbauer spectra of (1) aquohemerythrin and of (2) thiocyanato hemerythrin at 4.2°K and in magnetic field of 5 kgauss.

[2] Both low-spin and high-spin Fe(III) derivatives of hemoglobin (in which the iron atoms are definitely isolated) have Mössbauer spectra which are appreciably broadened at 4°K, even in the absence of a magnetic field, since at this temperature spin–lattice relaxation is slow (Lang and Marshall, 1966). Thus the absence of magnetic hyperfine broadening in methemerythrin must be due spin–spin interactions between neighboring iron atoms.

[3] To our knowledge no sample of pure (*i.e.*, >95%) oxyhemerythrin has ever been prepared. Fresh crystalline samples and even the protein in fresh laked blood contain at least 15–20% Fe(III) (Klotz *et al.*, 1957). Furthermore, molecular weight studies (Klotz and Keresztes-Nagy, 1963) almost always show some low molecular weight material, monomeric hemerythrin, whose presence indicates oxidation or blockage of the sulfhydryl group on the protein.

establish that the two iron atoms are interacting. Furthermore, they are in essentially identical magnetic environments, and the similarity in spectra of metal protein and model binuclear iron compounds provides strong evidence that the bridge formulation, $Fe–O_2–Fe$, for the active site of hemerythrin is correct.

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