Shih, A., and Eisenstadt, J., and Lengyel, P. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1599.

Stent, G. S., and Brenner, S. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 2005.

Active Site of Hemerythrin. Iron Electronic States and the Binding of Oxygen*

J. Lyndal York† and Alan J. Bearden

ABSTRACT: Hemerythrin, a molecular oxygen-binding nonheme protein, found in marine organisms, binds a single oxygen molecule per protein subunit containing two Fe atoms.

The nature of the oxygen binding and the electronic state of Fe in oxy, deoxy, and oxidized forms of the protein subunits has been investigated by Mossbauer spectroscopy, magnetic susceptibility measurements, and by chemical means. Oxidized (met) hemerythrin derivatives show a single high-spin Fe(III) ($S = {}^5/_2$) site for the two Fe atoms; these sites are then spin coupled to give molecular diamagnetism. In deoxyhemerythrin the single environment is high-spin Fe(II) ($S = 2$) with no evidence of spin coupling as shown by susceptibility measurements. The oxy form of the protein shows two Fe environments, a result in contrast to a symmetrical oxo bridge or superoxide anion model. The relation of these physical studies to other studies and a discussion of oxygen binding in hemerythrin is given.

Hemerythrin is a nonheme iron protein which serves as a reversible oxygen carrier in the red cells of the brachipods and the sipunculids. The mechanism of reversible oxygen binding is of great interest *vis a vis* the oxygen binding of the heme proteins.

Klotz and coworkers (Klotz and Klotz, 1955; Klotz *et al.*, 1957; Klotz and Keresztes-Nagy, 1963; Keresztes-Nagy and Klotz, 1965; Groskopf *et al.*, 1966; Klapper and Klotz, 1968; Langerman and Klotz, 1969) have characterized the macromolecular properties of the protein.

Hemerythrin can be dissociated into eight subunits by sulfhydryl reagents; each subunit has a molecular weight of 13,500, contains two Fe atoms, and is capable of binding a single molecule of oxygen (Love, 1957; Boeri and Ghiretti-Magaldi, 1957). There is but a single cysteine residue per monomer unit; this residue is involved in subunit binding, not iron binding (Keresztes-Nagy *et al.*, 1965), and there is no "acid-labile" sulfide in contrast to the ferredoxins (Fry and San Pietro, 1962).

The oxidation state of the iron in various forms of hemerythrin has been in dispute as has the mechanism of reversible oxygen binding. Kubo (1953) on the basis of magnetic susceptibility measurements proposed a ferrous state for the iron in the oxygen-bound complex. Other workers have presented data which suggest a ferric–peroxy complex (Klotz and Klotz, 1955; Klotz *et al.*, 1957). These latter workers assayed the content of bivalent iron by color formation with *o*-phenanthroline after liberation of the iron from the protein by acid treatment. No ferrous iron was released from oxygenated hemerythrin. Deoxyhemerythrin was shown to contain 2 moles of ferrous and 0.4 mole of ferric iron per mole of protein. The conclusion was drawn that the ferrous deoxyhemerythrin was converted into a ferric oxyhemerythrin by oxygen binding. The rational and conclusions of these experiments have been challenged (Williams, 1955; Boeri and Ghiretti-Magaldi, 1957) on the basis that rapid oxidation of ferrous ion occurs at pH values below 3 or 4 (Roaf and Smart, 1923). Thus the question of the oxidation state of iron in the oxygenated hemerythrin and the nature of the oxygen iron complex has not been resolved although reasonable answers to several of these objections have been given (Keresztes-Nagy *et al.*, 1965). These workers have more recently presented Mossbauer and circular dichroic spectral data which has been interpreted as supporting the earlier conclusion of a ferric–peroxy complex for the oxygen compound (Garbett *et al.*, 1969; Okamura *et al.*, 1969).

In this paper nuclear γ-ray resonance spectroscopy (Mossbauer effect) and measurements of magnetic susceptibility are employed in order to investigate the active site of hemerythrin. A preliminary abstract of the work has appeared (York and Bearden, 1968).

Experimental Section

Preparation of Hemerythrin and Derivatives. The method of purification of hemerythrin was essentially that previously described (Florkin, 1933; Klotz *et al.*, 1957). The worms were cut; the contents squeezed out and filtered through cheesecloth. The red cells were washed free of white cells and a brown coagulum by centrifugation and resuspended in fresh sea water. To the packed red cells was added an equal

* From the Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee, and the Donner Laboratory, University of California, Berkeley, California. *Received June 22, 1970.*

† Present address: Department of Biochemistry, University of Arkansas School of Medicine, Little Rock, Arkansas. To whom correspondence should be addressed.

volume of cold distilled water. The cells were then laked by two cycles of freezing and thawing. The resulting mixture was clarified by centrifugation at 100,000*g* for 1 hr. The supernatant was crystallized from a 20% ethanol–water mixture at 4°. Three such crystallizations were carried out with intermediate solvation in 0.5 M sodium phosphate buffer, pH 7.0. The iron to protein ratio of material prepared in this manner could not be improved by chromatography on Sephadex G-200.

The material prepared from either ethanol crystallization or Sephadex chromatography had an iron content of 0.81% and an extinction coefficient of 3400 cm l. mole^{-1} at 330 mμ. These values are identical with those reported by others (Klotz *et al.*, 1957; Keresztes-Nagy and Klotz, 1965). The 280/330 ratio was 5.64 as previously reported (Love, 1957).

The methemerythrin and its derivatives were prepared by passing the purified oxyhemerythrin through a 2 × 10 cm column of Dowex 1-X8 which had been previously equilibrated with the sodium salt of the anion whose methemerythrin complex was desired. Concerted oxidation and complex formation occurred on the column. The desired complex was eluted from the column with distilled water and crystallized from 20% aqueous ethanol. Aquomethemerythrin was prepared by dialysis of the methemerythrin chloride against 0.05 M phosphate, pH 7.0. The conversion was followed spectroscopically (Keresztes-Nagy and Klotz, 1965). The crystalline material was collected by centrifugation and resuspended in distilled water to wash out ethanol. For the Mossbauer experiments, the crystalline derivatives were packed in Mossbauer cells by centrifugation.

It was found that at concentrations of hemerythrin which were necessary for Mossbauer experiments, complete deoxygenation of oxyhemerythrin by alternate evacuation and flushing with nitrogen did not yield a completely colorless preparation. A brownish color was associated with deoxyhemerythrin prepared in this manner. Deoxyhemerythrin for which Mossbauer parameters are reported was prepared by adding a tenfold molar excess of sodium dithionite to crystalline oxyhemerythrin suspended in a small volume of 20% ethanol. The red color of the oxygenated protein disappeared immediately, leaving colorless crystals of hemerythrin. All procedures were carried out at 5° and in a nitrogen atmosphere. To determine if dithionite reduction gave rise to any artifacts in the Mossbauer spectrum, deoxyhemerythrin was also prepared by deoxygenation of the sample with glucose oxidase. Glucose oxidase (1 mg) (Sigma Chemical Co.) was added to a 1-ml solution of oxyhemerythrin (80 mg/ml) which had been dialyzed against 0.1 M phosphate, pH 7.0, and 0.05 M glucose. The deoxygenation reaction was run in the sealed Mossbauer cell. The reaction mixture was frozen in the cell with liquid nitrogen and the Mossbauer parameters determined after all the oxygen was removed as evidenced by conversion of the deep red color of the oxyhemerythrin into the pale yellow of the deoxyhemerythrin.

Redox Titration. The number of ferricyanide-oxidizable iron atoms per molecule of oxyhemerythrin was determined by a spectrophotometric titration of oxygenated hemerythrin with potassium ferricyanide. Attempts to carry out the titration in the range of 20–30° were unsuccessful because of protein denaturation which resulted in cloudy solutions and errant optical density readings. The following procedure was therefore adopted. Aliquots of 0.01 M potassium ferricyanide solution were added to tubes containing 0.88 μmole of hemerythrin iron as determined by iron analysis, in a solution made 0.05 M in phosphate, pH 7.0, and 0.5 M in potassium chloride. The tubes were allowed to sit at 4° for 24 hr. Since chloride will complex only with the oxidized hemerythrin, the course of titration could be followed by observing the decrease in optical density of the 500-mμ band associated with the oxygenated hemerythrin (Keresztes-Nagy and Klotz, 1965).

Mossbauer Spectroscopy. The Mossbauer spectrometer has been previously described (Bearden *et al.*, 1965; Moss *et al.*, 1968). Due to the time span of the investigation and the use of two spectrometers three different sources were used. The sources were 20 mCi of ^{57}Co in a copper matrix; 45 mCi of ^{57}Co in a chromium matrix; and 40 mCi of ^{57}Co in a palladium matrix. The isomer shifts were reported for the source with which the data were acquired and also reported with respect to the center lines of an iron foil standard so that comparisons can be made. The detector was a proportional counter with a fill gas of 95% Kr–5% N_2. Data were collected in 200 or 256 channels of a multichannel analyzer.

The standard sample used for the Mossbauer measurements contained 43 μmoles of naturally abundant iron with a sample thickness no greater than 10 mm. Since unenriched samples were used, this iron content corresponds to approximately 1 μmole of ^{57}Fe. The crystalline samples were packed in the cell by centrifugation and sealed under a He atmosphere. The latter operation is necessary for temperature equilibration of the samples in the region of the boiling point of liquid helium. The Mossbauer cell is a plastic vial (Polyvial, size A, obtained from The Chemical Rubber Co.).

Mossbauer spectra were obtained in from 6- to 10-hr running time with naturally enriched hemerythrin samples. Some attempts were made to study the possible enrichment of hemerythrin with ^{57}Fe in order to increase the suitability for Mossbauer spectroscopy by studying the uptake of ^{59}Fe by worms placed in a medium high in ^{59}Fe. Contrary to an earlier report (Schulman, 1957), no incorporation of ^{59}Fe could be seen although the worms did scavenge ^{54}Mn, present as a radioactive impurity in the ^{59}Fe, selectively. Chemical exchange of the iron in hemerythrin has not proven feasible.

Magnetic Susceptibility. The room-temperature magnetic-susceptibility measurements were made by using a Gouy balance. Two experimental arrangements were used: the first employed a Varian 12-in. electromagnet operating at 10.27 kG with a gap of 2.54 in., the second used a Varian 12.5-in. electromagnet operating at a field of 13 kG with a gap of 1 in. An Ainsworth 24 N balance with a reproducibility and a sensitivity of 0.01 mg was employed in both series of experiments. The cell was constructed of a Wilmad precision bore pyrex tube (Wilmad Glass Co., Buena, N. J. 08310) with an i.d. of 11.15 mm. A flat Plexiglas bottom was glued on with silastic. This tube was used with the 1.5-in. magnet and was filled to a height of 24 cm. The field at this point was negligible as determined with a gauss meter. The cell used with the large magnet had an i.d. of 12.7 mm and was filled to a height of 27 cm. The bottom 4 in. of tube containing sample was between the magnet pole faces. The cells were calibrated using triple glass-distilled water *vs.* air and were cross-calibrated using nickel chloride and ferrous ammonium sulfate. The experiments were carried out over a 3-month period using three different preparations of hemerythrin. All measure-

TABLE I: Mossbauer Parameters for Hemerythrin and Its Derivatives at 77°K.

Sample		Quadrupole Splitting ΔE (mm/sec)	Measured Isomer Shift δE (mm/sec)	δE Referenced to Iron[d]
Deoxyhemerythrin, (dithionite reduced)		2.77 ± 0.02	+1.01 ± 0.02[a]	+1.19 ± 0.02
Oxygenated hemerythrin	(i)	1.86 ± 0.04	+0.24 ± 0.04[b]	+0.47 ± 0.04
	(ii)	1.04 ± 0.04	+0.24 ± 0.04	+0.47 ± 0.04
Methemerythrin, azide, fluoride, or thiocyanate		1.76 ± 0.02	+0.60 ± 0.02[c]	+0.45 ± 0.02

[a] Relative to ^{57}Co in palladium; −0.185 relative to iron. [b] Relative to ^{57}Co in copper; −0.226 relative to iron. [c] Relative to ^{57}Co in chromium; 0.152 relative to iron. [d] Muir *et al.* (1966).

ments were made on solutions of hemerythrin made up in 0.1 M phosphate buffer at pH 7.5. Solutions were made from crystalline hemerythrin which had been extensively dialyzed against 10^{-3} M EDTA. The volume susceptibility was based on the difference in weight change of the hemerythrin as compared to the dialysis solution. The calculated molar susceptibility is based on the total iron content as determined by the *o*-phenanthroline method.

Iron Analysis. Iron concentrations were determined colorimetrically (Fortune and Mellon, 1938) after ashing the sample in nitric–perchloric acid (Ballentine and Burford, 1957).

Hemoglobin. CO-hemoglobin and CN-hemoglobin were prepared from human blood. Red blood cells were prepared by several washes with isotonic saline and then lysed. CO-hemoglobin was prepared by dialyzing O_2-hemoglobin against a CO-saturated 0.1 M borate buffer, pH 9.0, containing 0.001 M sodium dithionite. Stirring was by CO bubbling. Dithionite was removed by further dialysis against the CO-saturated borate buffer. The methemoglobin CN was prepared by addition of excess ferricyanide to a solution of oxyhemoglobin in 0.1 M borate, pH 9.0, containing 0.01 M NaCN. Ferricyanide and ferrocyanide were removed on a Sephadex G-25 column.

Infrared Spectra. Hemerythrin azide was examined on a Perkin-Elmer 521 spectrometer using a thermostated cell of 0.02-mm path length and a protein concentration of 100 mg/ml. The hemerythrin azide had been crystallized from 20% ethanol and thus contained no excess azide.

Results

Table I contains a summary of the experimentally determined Mossbauer parameters for hemerythrin and hemerythrin derivatives. The isomer shift, δE, is a measure of the s-electron density and can be related to the covalency as the s-electron density is sensitive to screening by the 3d electrons (Walker *et al.*, 1961). The quadrupole splitting, ΔE, arises from an interaction of a nonzero electric field gradient at the Fe nuclear position with the nuclear quadrupole moment of the $I = {}^3/_2$ state of the ^{57}Fe nucleus. The quadrupole splitting and the isomer shift are measured in units of mm/sec; the reference for isomer shift is Fe metal. The Mossbauer spectra are shown in Figures 1–4. There was no significant temperature dependence on any of the spectra between 77° and 4.6 °K. Oxyhemerythrin from *Goldfingia gouldii* and *Dendrostrmum zostericulum* showed identical Mossbauer spectra.

Table II lists the results of the room-temperature measurements of magnetic susceptibilities for hemerythrin and hemerythrin derivatives. Measurements on deoxyhemerythrin, oxyhemerythrin, and methemerythrin thiocyanate are uncorrected for the diamagnetic contribution to the susceptibility of the protein; this correction has been made for hemoglobin cyanide included as a comparison by using an equivalent concentration of CO-hemoglobin in the measurement and

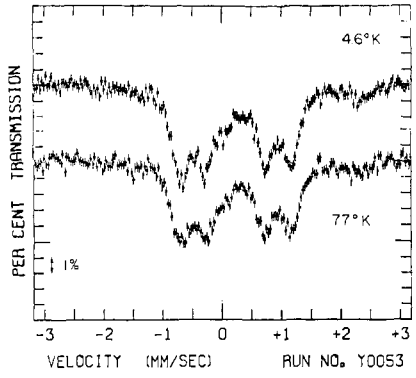

FIGURE 1: Mossbauer spectrum of oxygenated hemerythrin at 77° and 4.6°K.

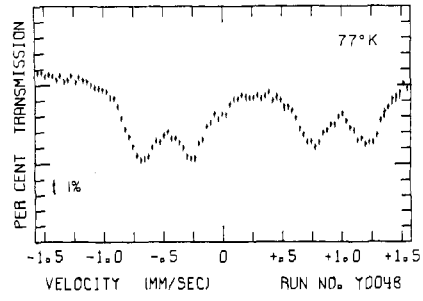

FIGURE 2: Mossbauer spectrum of oxygenated hemerythrin at 77°K with a twofold increase in energy resolution on the velocity axis.

TABLE II: Magnetic Susceptibility of Hemerythrin and Its Derivatives at 22°.

Compd	Iron Concentration (M)	ΔW Dialysis Solution − ΔW Hemerythrin (mg)	Field k (gauss)	Cell Constant ($\times 10^6$ cgs/mg)	X_M (10^6 cgs)
Deoxyhemerythrin	0.0060	−4.12	10.26	15.04	10,566[a]
	0.0059	−3.91	10.26	15.04	9,967[b]
	0.0050	−3.11	10.26	15.04	9,354[a]
	0.0058	−3.31	10.26	15.04	8,539[b]
	0.0039	−2.38	10.26	15.04	9,200[b]
					av 9,325 ± 562[e]
Oxyhemerythrin[c]	0.0140	−2.04	14.00	10.25	1,480
	0.0220	−2.71	14.00	10.25	1,264
	0.0220	−2.37	11.97	14.00	1,500
	0.0050	−0.35	10.26	15.04	1,053
	0.0160	−2.09	14.00	10.25	1,340
					av 1,328 ± 155[e]
Methemerythrin thiocyanate[d]	0.0077	−1.21	14.00	10.25	1,610
	0.0068	−0.93	14.00	10.25	1,400
	0.0108	−1.23	14.00	10.25	1,175
	0.0140	−1.40	14.00	10.25	1,025
	0.0140	−1.34	13.05	11.80	1,130
					av 1,268 ± 189[e]
Hemoglobin cyanide	0.0065	−1.65	14.00	10.25	2,620
		−1.45	14.00	10.25	2,290
					av 2,455[f]

[a] Deoxygenated with 10 mg of glucose oxidase in 0.1 M phosphate, pH 7.0, containing 0.05 M glucose. [b] Deoxygenated with 100 mg of $Na_2S_2O_4$ in 25 ml of 0.1 M phosphate, pH 8.0. [c] In 0.1 M phosphate, pH 8.0. [d] In 0.1 M phosphate, pH 7.5, and 0.02 M in NaSCN. [e] Not corrected for diamagnetic contribution of protein. [f] Calculation includes diamagnetic correction. This was done by using an equivalent concentration of CO-hemoglobin and subtracting the weight change of the methemoglobin from the weight change of the CO-hemoglobin.

then subtracting the weight change of the methemoglobin from the weight change of the CO-hemoglobin.

In addition to the results obtained by Mossbauer spectroscopy and measurements of the magnetic susceptibilities of these materials, electron paramagnetic resonance investigations of all derivatives were made in order to correlate the other results. Beinert *et al.* (1962) have reported a sharp electron paramagnetic resonance at $g = 2.02$; this signal was also observed in the earliest studies in this work although application of the electron paramagnetic resonance quantita-

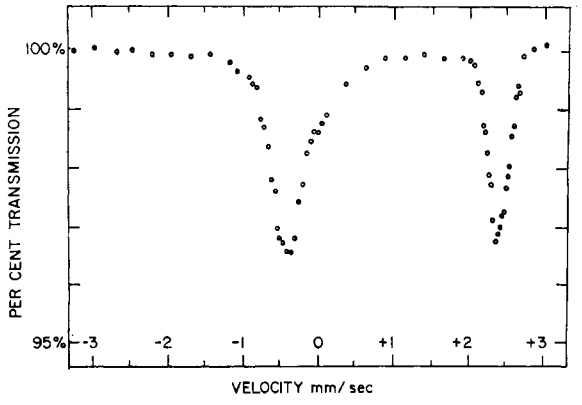

FIGURE 3: Mossbauer spectrum of deoxygenated hemerythrin at 77°K. The sample is dithionite-reduced hemerythrin.

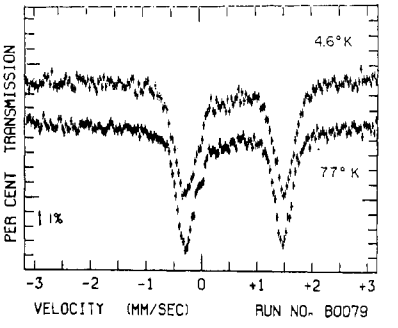

FIGURE 4: Mossbauer spectrum of methemerythrin azide at 77° and 4.6°K.

tion methods of Palmer (1967) showed clearly that this electron paramagnetic resonance signal was due to only 10–15% of the Fe present assuming that each Fe contributed a single unpaired electron to the signal. The line width of the signal indicates that it is not due to a free radical present in either the protein or the buffer solution. Denaturation of hemerythrin with 8 M urea or by acid precipitation produced other nonquantitative electron paramagnetic resonance signals with g values of 2 and 4.3. These electron paramagnetic resonance spectra were taken with a Varian E-3 at microwave power levels from 10 to 200 mW and at temperatures between −180° and room temperature.

Figure 5 indicates that oxygenated hemerythrin can be stoichiometrically oxidized by titration with ferricyanide in the presence of KCl to a form which is spectrally classifiable as methemerythrin chloride (Keresztes-Nagy and Klotz, 1965). The reaction is stoichiometric in that 1 mole of ferricyanide is required for each iron atom oxidized. The total amount of iron is known by chemical analysis; all of this iron is accounted for by the change in extinction at 500 nm as the oxidation proceeds. The rate of oxidation is slow; good stoichiometry is not obtained unless 8–12 hr at 4° is allowed for equilibrium.

Deoxyhemerythrin can be prepared by either dithionite treatment or by glucose–glucose oxidase deoxygenation; preparation by successive flushing of deoxyhemerythrin with N_2 gas at hemerythrin concentrations of 200–300 mg/ml did not produce samples free of methemerythrin as judged by Mossbauer spectroscopy.

The infrared spectrum of methemerythrin azide complex, in the N=N stretching region shows a band at 2046 cm^{-1} with a half-band width of 7.5 cm^{-1}.

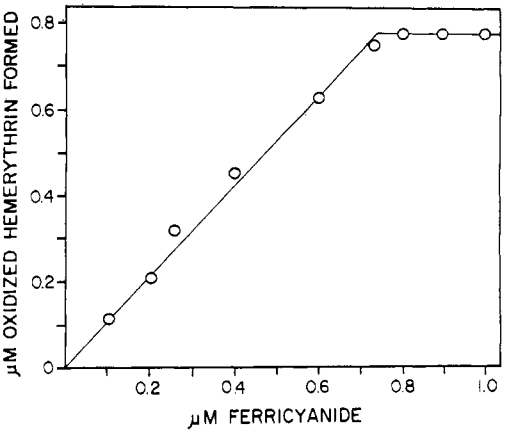

FIGURE 5: Titration of oxygenated hemerythrin with potassium ferricyanide in the presence of 0.5 M potassium chloride.

Discussion

The Mossbauer spectroscopic results point out that there are single environments for all the iron in both deoxyhemerythrin and in the oxidized derivatives of hemerythrin, but that these two classes of the protein have different iron environments. The iron environment in oxyhemerythrin is of two other types, each type being occupied by a single iron atom. The single iron environment in deoxyhemerythrin is clearly of the high-spin Fe(II) type; the large quadrupole splitting (2.8 mm/sec) being the characteristic signature of this ionization state (Fluck *et al.*, 1963). In addition, the room-temperature, magnetic-susceptibility data clearly indicate four unpaired electrons in deoxyhemerythrin. The single environment of all the iron in the oxidized forms of hemerythrin is less clear, but the assignment of high-spin Fe(III) states is suggested by the data. Magnetic-susceptibility measurements on several methemerythrin complexes between 77° and 4.6°K show no paramagnetism (T. H. Moss and J. L. York, 1970, in preparation) which suggests a spin coupling between the two iron atoms. The room-temperature magnetic data, which indicate one unpaired electron, are not in conflict with the concept of a spin coupled, $S = {}^5/_2$ state since similar magnetic results have been found for a binuclear iron orthophenanthroline complex (Khedekar *et al.*, 1967). Many model compounds of high-spin Fe(III) configuration have quadrupole splittings markedly less than the 1.7 mm/sec shown by the oxidized derivatives of hemerythrin (Fluck *et al.*, 1963), but highly distorted electronic surroundings, such as produced by the porphyrin ring for iron in ferriheme proteins, can cause quadrupole splittings of large magnitude (Lang and Marshall, 1966; Moss *et al.*, 1968). This analogy, of course, does not mean that there are any such ring structures in hemerythrin although multiple binding of the ring structure of histidine or another system could perhaps produce the effect. Recent chemical evidence indicates the coordination of two histidine residues per iron atom (Fan and York, 1969). The high-spin Fe(III) assignment is further substantiated by the results of the infrared study. The frequency of the N=N stretch for the methemerythrin azide is very close to that of free azide in water (2050 cm^{-1}) and is twice as narrow as the free-ion absorption. These findings suggest that the azide binding in methemerythrin is ionic in character and, therefore, consistent with the high-spin assignment. These N=N stretching frequencies are similar to those reported for the high-spin component of azide metmyoglobin and protoheme (McCoy and Caughey, 1970). These results corroborate the recent findings of others that the iron in methemerythrin is high-spin and antiferromagnetically coupled (Okamura *et al.*, 1969; Garbett *et al.*, 1969).

Addition of oxygen to deoxyhemerythrin modifies the iron environment of all the iron; *none* is left in the high-spin Fe(II) state. It is clear that there are *two* equally populated Fe states in oxyhemerythrin as was pointed out earlier (York and Bearden, 1968); however, evidence (Okamura *et al.*, 1969) has been convincingly presented which indicates that one of the two quadrupole pair is an artifact of oxidation and that in reality all the iron in oxyhemerythrin is in one environment. Fortunately, this conflict has been resolved by a more recent report (Garbett *et al.*, 1969) which concludes that there are two equally populated Fe states in oxyhemerythrin. Neither of the two iron environments in oxyhemerythrin are the same as the environments in either deoxyhemerythrin or in oxidized forms of the protein.

The fact that oxyhemerythrin can be oxidized stoichiometrically to the met form by ferricyanide strongly suggests that the hydroperoxide or superoxide (Garbett *et al.*, 1969) concept for oxygen binding in hemerythrin is inadequate. Certainly one would expect the potential of the iron-bound hydroperoxide or superoxide to be much more positive than that of the ferricyanide. From this viewpoint it would not

be expected that ferricyanide could oxidize the iron–hydroperoxide complex.

Although there is ample physical evidence from Mossbauer data and from measurements of magnetic susceptibility to support spin coupling between the two Fe atoms in each subunit of methemerythrin, there are experimental results which suggest that this coupling scheme may not operate in oxyhemerythrin. The Mossbauer data for oxyhemerythrin show *two* distinct iron environments. The large dissimilarity in the quadrupole splittings shown by the two pairs of lines in the Mossbauer spectra of oxyhemerythrin (1.86 mm/sec *vs.* 1.04 mm/sec) is hard to reconcile with a small distortion of ferric sites. Further investigation by Mossbauer spectroscopy with the advantages of applied magnetic field may give more data on this point. In addition, magnetic susceptibility measurements on the same sample over a 4–300°K temperature range would possibly show the nature of coupling between iron atoms in the hemerythrin subunits. As there are many other possibilities for reversible oxygen binding (McGinnety *et al.*, 1969) which are thermodynamically more favorable than the superoxide anion, and which involve a single Fe site, it is perhaps best to withhold final decision on this matter.

Acknowledgments

We wish to acknowledge helpful discussions with Janet K. Allen, J. L. Hoard, J. A. Ibers, T. H. Moss, and R. H. Sands. The authors are grateful to S. McCoy for performing the infrared spectroscopy on hemerythrin azide and to J. F. Weir and A. Gremellion for permitting us to use their magnetic-susceptibility apparatus. This investigation has been supported by grants-in-aid to J. L. York from the National Institutes of Health (GM-14191) and to A. J. Bearden from the National Science Foundation (GB 5458 and 13585) and from the U. S. Atomic Energy Commission through the Donner Laboratory. The second author (A. J. B.) has been partially supported through a Public Health Service Research Career Development award (1-K4-GM-24,494-01) from the Institute of General Medical Studies. Additional support to J. L. York from the National Science Foundation (GB-15755) is acknowledged.

References

Ballentine, R., and Burford, D. D. (1957), *Methods Enzymol. 3*, 1002.

Bearden, A. J., Hauser, M. G., and Mattern, P. L. (1965), *in* Mossbauer Effect Methodology, Vol. 1, Gruverman, I. J., Ed., New York, N. Y., Plenum.

Beinert, H., Heinen, W., and Palmer, G. (1962), Report of Symposium on Enzyme Models and Enzyme Structure, Brookhaven National Laboratory, Upton, Long Island, N. Y.

Boeri, E., and Ghiretti-Magaldi, A. (1957), *Biochim. Biophys. Acta 23*, 489.

Fan, C. C., and York, J. L. (1969), *Biochem. Biophys. Res. Commun. 36*, 365.

Florkin, M. (1933), *Arch. Int. Physiol. 36*, 247.

Fluck, E., Kerler, W., and Neuwirth, W. (1963), *Angew. Chem. 2*, 277.

Fortune, W. B., and Mellon, M. G. (1938), *Ind. Eng. Chem. Anal. Ed. 10*, 13.

Fry, K. T., and San Pietro, A. (1962), *Biochem. Biophys. Res. Commun. 9*, 5.

Garbett, K., Darnall, D. W., Klotz, I. M., and Williams, R. J. P. (1969), *Arch. Biochem. Biophys. 135*, 419.

Groskopf, W. R., Holleman, J. W., Margoliash, E., and Klotz, I. M. (1966), *Biochemistry 5*, 3779.

Keresztes-Nagy, S., and Klotz, I. M. (1965), *Biochemistry 4*, 919.

Keresztes-Nagy, S., Lazer, L., Klapper, M. H., and Klotz, I. M. (1965), *Science 150*, 357.

Khedekar, A. V., Lewis, J., Mobbs, F. E., and Weigold, H. (1967), *J. Chem. Soc., A*, 1561.

Klapper, M. H., and Klotz, I. M. (1968), *Biochemistry 7*, 223.

Klotz, I. M., and Keresztes-Nagy, S. (1963), *Biochemistry 2*, 445.

Klotz, I. M., and Klotz, T. A. (1955), *Science 121*, 477.

Klotz, I. M., Klotz, T. A., and Fiess, H. A. (1957), *Arch. Biochem. Biophys. 68*, 284.

Kubo, M. (1953), *Bull. Chem. Soc. Jap. 26*, 244.

Lang, G., and Marshall, W. (1966), *J. Mol. Biol. 18*, 385.

Langerman, N. R., and Klotz, I. M. (1969), *Biochemistry 8*, 4746.

Love, W. E. (1957), *Biochim. Biophys. Acta 23*, 465.

McCoy, S., and Caughey, W. S. (1970), *Biochemistry 9*, 2387.

McGinnety, J. A., Payne, N. C., and Ibers, J. A. (1969), *J. Amer. Chem. Soc. 91*, 6301.

Moss, T. H., Bearden, A. J., Bartsch, R. G., Cusanovich, M. A., and San Pietro, A. (1968), *Biochemistry 7*, 1591.

Muir, A. H., Jr., Ando, K. J., and Coogan, H. M., Ed. (1966), *in* Mossbauer Effect Data Index: 1958–1965, New York, N. Y., Wiley-Interscience, p 26.

Okamura, M. Y., Klotz, I. M., Johnson, C. E., Winter, M. R. C., and Williams, R. J. P. (1969), *Biochemistry 8*, 1951.

Palmer, G. (1967), *Methods Enzymol. 10*, 603.

Rill, R. L., and Klotz, I. M. (1970), *Arch. Biochem. Biophys. 136*, 507.

Roaf, H. F., and Smart, W. A. N. (1923), *Biochem. J. 17*, 579.

Schulman, M. P. (1957), *Biol. Bull. 118*, 361.

Walker, L. R., Wertheim, G. K., and Jaccarino, V. (1961), *Phys. Rev. Lett. 6*, 98.

Williams, R. J. P. (1955), *Science 122*, 558.

York, J. L., and Bearden, A. J. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 781.