Mossbauer Spectroscopic Evidence for Low-Spin Iron in Dehydrated Metmyoglobin*

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ABSTRACT: Enriching myoglobin in the Mössbauer isotope ^{57}Fe permits high sensitivity Mössbauer spectroscopy of the protein. Protein enrichment has been accomplished by combining enriched protoheme (92.8% ^{57}Fe) with apomyoglobin. A prominent feature of the Mössbauer spectroscopy of the lyophilized Fe^{3+} protein is a line pair with quadrupole splitting of 2.04 ± 0.02 mm/sec, indicating that the iron is in the low-spin state ($S = {}^1/_2$). This result is in contrast to magnetic susceptibility measurements of the material in solution which indicate high-spin iron ($S = {}^5/_2$). Two possible mechanisms are suggested for stabilization of the low-spin state in the dehydrated protein. One previously proposed for anhydrous hemoglobins involves coordination of a second histidine (E7) to the iron; the other involves a subtle change in bonding at the iron site due to removal of water bound at the surface of the protein.

The temperature-dependent line broadening of the Mössbauer spectrum, observed over a range from 4.6 to 298°K, arises from magnetic interaction between the iron electrons and the nucleus, coupled with the temperature dependence of the electron spin-lattice relaxation time.

Recently Mössbauer spectroscopy has become available as a biochemical research tool. Mössbauer studies provide information complementary to that derived from other physical and chemical techniques. One of the most useful Mössbauer isotopes, ^{57}Fe, can be incorporated into many biomolecules either by substitution chemistry or by growing an organism on ^{57}Fe-enriched nutrients (Bearden *et al.*, 1965a). Biomolecules of special interest to us have been the iron porphyrin compounds; a summary and interpretation of data obtained by Mössbauer spectroscopy on ^{57}Fe-enriched hemin chlorides and dipyridine hemes has recently been published (Bearden *et al.*, 1965b). This paper will present a method of preparing ^{57}Fe-enriched sperm whale metmyoglobin and give results of a systematic study by Mössbauer spectroscopy of this hemeprotein.

In the past decade much new information has been obtained on the structure and properties of metmyoglobin. The elucidation of the three-dimensional structure of metmyoglobin by X-ray crystallography has shown the heme iron bound on one side to a protein imidazole group (F8) (Kendrew *et al.*, 1960; Kendrew, 1963). Another imidazole group (E7) is in close proximity on the opposite side of the heme plane and is in a position which would permit hydrogen bonding to an iron-bound water molecule. Also, the iron atom was found to be out of the plane of the porphyrin ring toward the F8 imidazole. This information provides a starting point for interpretation of Mössbauer data: Mössbauer spectroscopy yields detailed information on the electronic configuration near the iron atom (Wertheim, 1964).

Three relative energy parameters are measured by Mössbauer spectroscopy of paramagnetic or diamagnetic materials. The nuclear isomer shift is proportional to the total s-electron density at the nuclear position. The s-electron density is strongly influenced by differences in shielding due to changes in the outer electronic shells (3d in iron compounds) so that the isomer shift is a sensitive function of the electronic configuration (Walker *et al.*, 1961). In particular, measurements of the isomer shift can give information on delocalization of valence electrons. The electric quadrupole splitting is a measure of the electric field gradient at the nucleus. The splitting is due to the interaction of the electric quadrupole moment of the ^{57}Fe excited nuclear state with the derivatives of the electric field. The magnitude of an electric field gradient at a nucleus depends on the atomic electronic configuration and the ligand or crystal field surroundings. Magnetic hyperfine splitting of Mössbauer lines normally associated with spin-ordered materials has also been observed in paramagnetic substances. To see the splitting in paramagnets it is necessary that the spin-lattice relaxation time be

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longer than h/E_H, where h is Planck's constant and E_H is the energy splitting of the hyperfine lines. h/E_H is 10^{-8}–10^{-9} sec in typical paramagnets (Wertheim and Remeika, 1964). The magnetic splitting with 0 external field will thus depend on the configuration of the atomic electrons and the interaction of the iron atom with the crystal lattice and other paramagnetic ions.

Mössbauer resonance spectroscopy has been previously applied to the study of hemoglobin and myoglobin. However these prior studies have been made with proteins containing natural or only slightly enriched iron (Gonzer and Grant, 1963, 1964; Maling and Weissbluth, 1964). There is a considerable advantage in seeking high (>50%) enrichment of iron proteins for Mössbauer investigations. This permits determination of the isomer shifts and splittings to small parts of a line width, with sufficient accuracy to draw conclusive information. A detailed explanation presenting the parameters important to a spectroscopic run are included in the Appendix. The metmyoglobin reported on in this work has been enriched to 92.8% ^{57}Fe by the techniques detailed below.

Experimental Section

Preparation of Protoporphyrin IX. Protoporphyrin IX was prepared from hemin by the method of Ramsey (1953) with certain modifications. The crude protoporphyrin (6.1 g) obtained from 7.8 g of hemin (supplied by L. Light and Co.) was dissolved in 300 ml of 2% ammonium hydroxide followed by the addition of 52 ml of 30% aqueous disodium tartrate; no precipitate formed at this point on standing, but the addition of 23 g of disodium tartrate as a solid did effect precipitation. The precipitate was dissolved in 150 ml of 2% aqueous ammonium hydroxide and 30 ml of saturated aqueous disodium tartrate was added; the solution was allowed to stand at 0° for 45 min. A precipitate was collected and the reprecipitation process was repeated two more times. The resultant precipitate was mixed with 240 ml of 2% aqueous acetic acid and allowed to stand for 24 hr. As the precipitate was still appreciably soluble in water, 7 ml of glacial acetic acid was added to the mixture to convert the remaining salt to the acid form. After 24 hr the precipitate was collected, washed thoroughly with water, and dried under vacuum at 50° over phosphorus pentoxide. The dried material was dissolved in 75 ml of formic acid (97–100%). On the addition of 525 ml of anhydrous ether, a precipitate formed immediately. The precipitate was washed with ether and water followed by drying under vacuum at 50°; yield 2.6 g. Both electronic and nmr[1] spectra of the product in pyridine were as expected for protoporphyrin IX when compared with spectra for an authentic sample of the dimethyl ester (W. S. Caughey and J. L. York, 1963, unpublished; Caughey and Koski, 1962).

Anal. Calcd for $C_{34}H_{34}N_4O_4$: C, 72.57; H, 6.09; N, 9.97. Found: C, 72.80; H, 6.35; N, 9.81.

Preparation of Protoporphyrin IX Iron(III) Chloride Enriched to 92.8% with ^{57}Fe. A solution of ferrous acetate was prepared by refluxing glacial acetic acid (15 ml) under nitrogen in the presence of 65 mg of iron powder containing 92.8% ^{57}Fe (supplied by Oak Ridge National Laboratory). The solution was added slowly to a refluxing solution of protoporphyrin IX (300 mg) and sodium chloride (300 mg) in glacial acetic acid (250 ml) under nitrogen. After 10 min, 350 ml of hot aqueous sodium chloride (saturated at room temperature) was added followed by the addition of 200 ml of water; the mixture was allowed to stand at room temperature for 12 hr. The precipitate which resulted was collected by filtration, washed with 30% aqueous acetic acid and water until no chloride was detected in the washings, and dried under vacuum at 50°.

The protohemin chloride was isolated from several other products by partition chromatography on Celite (Caughey and York, 1962). The mobile and stationary phases were prepared by allowing a mixture of 2.5 l. of pyridine, 1.25 l. of chloroform, 1.25 l. of water, and 125 ml of isooctane to stand until the upper and lower phases were clear. The upper phase (250 ml) was shaken with Celite (300 g, Johns Manville No. 545 acid-washed) in a closed flask until the Celite appeared fluffy and did not stick to the surface of the flask. The wetted Celite was suspended in the lower phase and added to a column (5 × 50 cm) with stirring to eliminate air pockets; the Celite was further settled by passing the lower phase through the column. A small amount of sand was then layered on the top of the column. The crude protohemin was dissolved in the lower phase (50 ml) and added to the column. Elution with the lower phase developed five distinct zones on the column; the fastest moving zone, red-brown, contained the desired product and the eluate fraction for this zone was evaporated to dryness under vacuum. The dried residue was extracted with a mixture of pyridine (0.6 ml) and chloroform (1.3 ml). The extract and chloroform (1.5 ml) used to wash the flask and the filter were added to a refluxing mixture prepared from 0.5 ml of saturated aqueous sodium chloride, 0.4 ml of concentrated hydrochloric acid, and 9.6 ml of glacial acetic acid. Chloroform evaporated rapidly from the hot mixture which was allowed to stand open to the atmosphere for 12 hr at room temperature. A precipitate was recovered by filtration which was then washed with 30% aqueous acetic acid, with hot distilled water until there was no chloride in the washings, and with small amounts of ethanol and ether, and was dried under vacuum at 50°; yield 88 mg. In pyridine–0.05 N sodium hydroxide (1:1, v/v) with added sodium dithionite absorption spectra gave λ_{max} in mμ (A/A_α) of α, 555 (1.0), β, 525 (0.54), Soret, 419 (4.9); comparable data were obtained with protohemin chloride unenriched in ^{57}Fe.

Anal. Calcd for $C_{34}H_{32}N_4FeClO_4$: C, 62.23; H, 4.95. Found: C, 61.91; H, 5.18.

Preparation of Apomyoglobin. Apomyoglobin was prepared according to the method of Teale (1957) as modified by Breslow (1964). Lyophilized salt-free myoglobin (1 g) (samples were kindly provided by Dr. F.

[1] Abbreviations used: nmr, nuclear magnetic resonance.

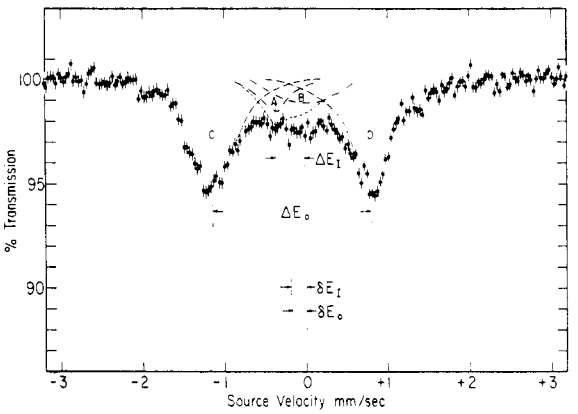

FIGURE 1: Mössbauer absorption spectrum of lyophilized metmyoglobin at 298°K.

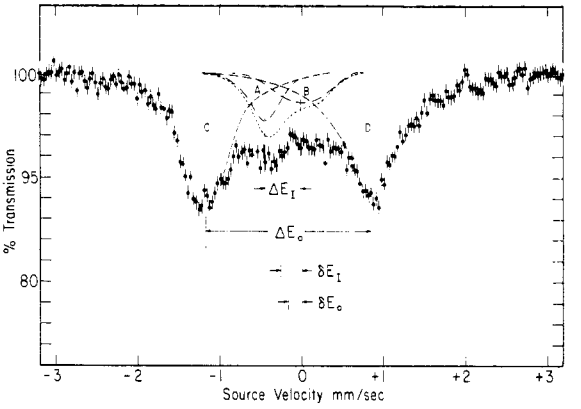

FIGURE 2: Mössbauer absorption spectrum of lyophilized metmyoglobin at 228°K.

R. N. Gurd; others were obtained from Mann Research Laboratories) was dissolved in 20 ml of water containing 20 mg of potassium ferricyanide; the solution was dialyzed *vs.* five changes of water. Glass-distilled water was used in the preparation of apomyoglobin and [^{57}Fe]metmyoglobin. A small amount of precipitate was removed from the dialyzed solution by centrifugation. The pH of the supernatant was adjusted to 1.5 with 1 N hydrochloric acid and diluted with water to a protein concentration of *ca.* 1% followed immediately by extractions at 4° with an equal volume of 2-butanone, and then two more extractions with half-volumes of 2-butanone. The protein solution, now only lightly colored, was dialyzed *vs.* three changes of dilute aqueous sodium bicarbonate (50 mg/l.), three changes of 1×10^{-4} M disodium EDTA, and five changes of water, and was concentrated by evaporation through the dialysis membrane to one-half the original volume. After removal of a small amount of precipitate by centrifugation, the solution gave an extinction coefficient of 999 ml g^{-1} cm^{-1} at 230 mμ.

Recombination of Apomyoglobin with ^{57}Fe Enriched Protohemin. The recombination procedure followed was that of Breslow (1964) with some modification (E. Breslow, 1965, personal communication). A quantity of [^{57}Fe]protohemin chloride 10% in excess of that required for equimolar combination with apomyoglobin (in this case about 20 mg) was wet with 0.1 ml of 1 N sodium hydroxide and immediately dissolved in borate buffer at pH 9.2 to a final concentration of *ca.* 1 mM. The borate buffer was prepared by dissolving 6.4 g of sodium hydroxide and 19.8 boric acid in 1 l. of water. The globin solution was diluted with borate buffer to a protein concentration of about 1% followed by the addition of the hemin solution with gentle stirring and dialysis *vs.* six changes of water. The dialyzed solution was centrifuged to remove some precipitate; the supernatant was lyophilized; yield 475 mg. A mean residue rotation, $[m']_{233}$, of -8600 was obtained for this product in water by Mr. A. J. Veros and Dr. K. D. Hardman in the laboratory of Dr. F. R. N. Gurd and agrees well with reported values for natural metmyoglobin under similar conditions; Breslow *et al.* (1965) reported a value of -8630.

Mössbauer Spectroscopy. The precision cam, constant acceleration Mössbauer spectrometer allows data to be taken over 6–20 hr in semiautomatic fashion. A 4 Mc ^{57}Co source in a copper matrix provides 0.5% statistical accuracy in this period, the exact running time depending on the sample absorber thickness and relative iron concentration. The protein samples were placed in sealed cells filled with 1 atm of He gas to provide effective heat exchange between the powder samples and the cell walls. The heat exchange gas is essential for thermal equilibrium. Without exchange gas the lyophilized powders would not cool to final temperatures for many hours. The Mössbauer spectra obtained with metmyoglobin are highly temperature dependent; runs without exchange gas produced completely spurious results. The spectra exhibited in this paper are confirmed by many repetitions. There was no apparent decomposition of the metmyoglobin samples either from radiation damage or in other steps of the procedure.

Results and Discussion

Preparation of Reconstituted Metmyoglobin ^{57}Fe Enriched. Iron (92.8% enriched with ^{57}Fe) was introduced into protoporphyrin IX *via* the classical ferrous acetate–acetic acid method of Warburg and Negelein (1932) (*cf.* Falk, 1964). Partition chromatography revealed the presence of four other products in addition to protohemin chloride. Band maxima in hemochromogen spectra for the other products were at shorter wavelengths by 2–5 mμ from those of the hemochromogen of protohemin indicating at least one vinyl group was no longer intact; no further attempts to characterize these products were made. The Mössbauer parameters of protohemin chloride and their implications have been discussed in a previous paper (Bearden *et al.*, 1965b). Combination of the hemin with apomyoglobin following the methods described above (Breslow, 1964; E. Breslow, 1965, personal communication) gave a product

TABLE I: Mössbauer Parameters[a] in Millimeters per Second for Lyophylized Myoglobin.

	Outer Pair of Lines					Inner Pair of Lines				
			Line Width (±0.1 mm/sec)					Line Width (±0.1 mm/sec)		
Temp (°K)	ΔE_0 (±0.02 mm/sec)	δE_0[b] (±0.02 mm/sec)	C	D	Relative Areas	ΔE_I (±0.04 mm/sec)	δE_I (±0.04 mm/sec)	A	B	Relative Areas
298	1.96	−0.18	0.6	0.5	*Ca.* equal	0.36	−0.20	0.4	0.9	*Ca.* equal
228	2.02	−0.18	0.7	0.8	*Ca.* equal	0.44	−0.26	0.4	0.8	*Ca.* equal
77	2.04	−0.12	0.9	1.4	*Ca.* equal	0.38	−0.08	0.4	0.9	*Ca.* equal
4.6			Broadened beyond recognition			0.44	0.0	0.5	0.5	*Ca.* equal

[a] Parameters are given as labeled in the figures. The resolution of the spectrometer is ±0.01 mm/sec. Additional errors are due to uncertainty in resolving overlapping curves. [b] Isomer shifts include the effect of the temperature difference between source and absorber. The source was at 298°K.

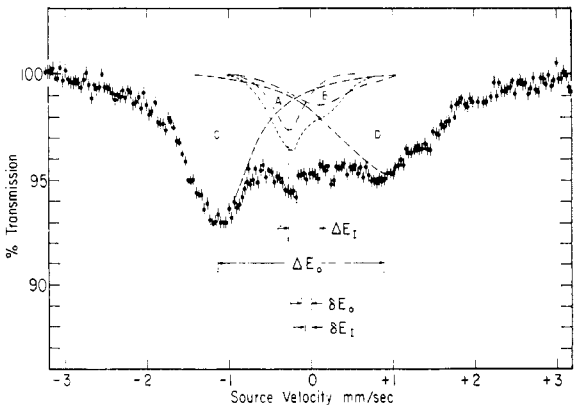

FIGURE 3: Mössbauer absorption spectrum of lyophilized metmyoglobin at 77°K.

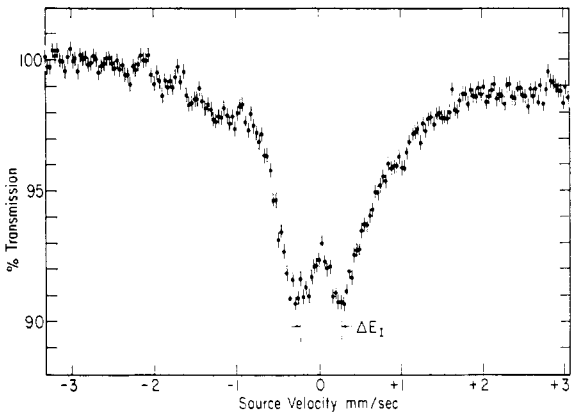

FIGURE 4: Mössbauer absorption spectrum of lyophilized metmyoglobin at 4.6°K.

in about 85% yield which in terms of absorption spectra and optical rotatory dispersion data was comparable to native metmyoglobin. Also, prior to combining apomyoglobin with ^{57}Fe-enriched hemin, the recombination method was checked in several recombination experiments carried out with natural hemin. Among the products obtained only slight differences in wavelengths (±2 mμ) and intensities of absorption maxima were found; $[m']_{233}$ values obtained for these preparations were −8400, −8800, and −7000. The origin of the small differences observed in reconstitution products, all of which were apparently handled experimentally in the same way, was not clear.

Mössbauer Spectroscopy. The Mössbauer spectra of lyophilized metmyoglobin at four sample temperatures are shown in Figures 1–4. The resolution of these curves is also shown in the figures and quantitatively in Table I. The main features are the existence of an outer and inner pair of lines. One member of the inner pair (marked B in Figures 1–4) is broadened from 298 to 30°K, while the outer pair (marked C and D) show a similar effect only below 228°K. At 4.6°K (the measured temperature of the sample cell with the liquid helium reservoir filled) the outer pair of lines appears to break up into very diffuse lines, while the inner pair becomes symmetric. A 4.6°K spectrum taken over a wide velocity (±6 mm/sec) scan is shown in Figure 5.

From the widely split (1.96 mm/sec) pair of quadrupole lines in the spectrum of lyophilized metmyoglobin at 298°, we infer that the iron is in the low-spin Fe^{3+} state and offer two possible mechanisms to explain the conversion of the high-spin iron in aqueous metmyoglobin solution (Taylor, 1939; Theorell and Ehrenberg, 1951) to the low-spin form of the dried material. The chemical conclusions can be drawn from the 298°K spectrum alone; the physical basis of the temperature dependence of the spectrum will be discussed separately.

The quadrupole splitting of the wide pair of lines is compatible with high-spin Fe^{2+} or low-spin Fe^{3+}. The

other two alternatives, low-spin Fe^{2+} and high-spin Fe^{3+}, have spherically symmetric electron configurations, and exhibit only small quadrupole splittings (0–1.0 mm/sec) due to distortion of this configuration by asymmetric ligand fields. These two possibilities can thus be eliminated.

The small negative isomer shift (−0.18 mm/sec) observed casts doubt on the possibility of having high-spin Fe^{2+}, even if a conceivable mechanism can be imagined for reduction of metmyoglobin on drying. Generally, the addition of a sixth d-electron greatly increases the shielding of the s-electrons and thus decreases the s-electron density at the nucleus. This leads to isomer shifts on the order 1.0–1.5 mm/sec in high-spin Fe^{2+} compounds. In cases where iron electrons are delocalized to surrounding ligands it is possible for iron compounds which are formally divalent to have small negative isomer shifts. However, it is only in the low-spin case, where three iron d-orbitals are filled, that iron can be expected to act as a strong donor of d-electrons. In the high-spin Fe^{2+} state the donor properties of the iron will be much weaker, and the isomer shift should be large and positive. Thus, it is unlikely that the wide lines represent high-spin Fe^{2+}.

Only the possibility that the iron is in the low-spin Fe^{3+} state in lyophilized metmyoglobin remains. This result is surprising in the sense that aqueous solutions of metmyoglobin are well known to contain high-spin iron (Taylor, 1939; Theorell and Ehrenberg, 1951). However, methemoglobin has been reported to undergo a reversible change in spin state on drying. Keilin and Hartree (1952) observed that the optical absorption of a film of the dried material shows a characteristic two-banded (567 and 534 mμ) parahematin spectrum instead of the acid methemoglobin spectrum with the characteristic band maxima near 500 and 630 mμ. Later Havemann and Haberditzl (1959) reported magnetic susceptibility data for dried methemoglobin which were consistent with low-spin iron. It is, therefore, reasonable to expect a similar change should take place on drying metmyoglobin. Such a change is clearly indicated by our Mössbauer spectrum. In work to be published later we have observed a similar wide split pair of lines in the spectrum of lyophilized methemoglobin, emphasizing the analogy between the effect of drying on the two proteins.

One interpretation of the change in iron coordination in lyophilized metmyoglobin was originally proposed by Haurowitz (1951) for hemoglobin and extended to methemoglobin by Keilin and Hartree (1952). These authors suggested that as the protein was dried the water bound to the iron was removed and a second histidine residue became coordinated to iron. Thus in metmyoglobin, binding of a second imidazole group (*i.e.*, E7) to iron could stabilize the low-spin state, in accord with our Mössbauer data. Of possible relevance here are our findings of a similar splitting (2.1 mm/sec) and temperature dependence in Mössbauer spectra of a *c*-type (Fe^{3+}) cytochrome from *Chromatium*. The iron in *c*-cytochromes has been found to be low spin in both Fe^{2+} and Fe^{3+} oxidation states and thus can be expected to have two strong-field ligands (histidine or other).

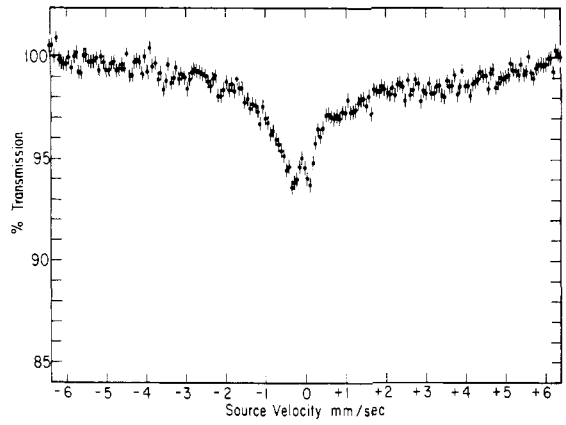

FIGURE 5: Mössbauer absorption spectrum of lyophilized metmyoglobin at 4.6°K showing diffuse absorption at high velocities.

An alternate explanation can also explain stabilization of the low-spin state, without great change in the distance between the iron and the E7 imidazole. In this case, water would not be removed from the iron atom but instead a water molecule bound to the E7 imidazole at the nitrogen (N_δ) most remote from iron would be removed, as shown in Figure 6. This histidine N_δ lies close to the surface, and in metmyoglobin crystals was found close to and presumably hydrogen bonded to a sulfate ion. We suggest that the removal of a water molecule from this nitrogen would result in a stronger (and shorter) N–H bond (a) and a weaker (and longer) O–H bond (b). Furthermore, the Fe–O bond would become shorter (and stronger) with a concomittant stabilization of the low-spin state for Fe^{3+}. One could then also expect the Fe atom to be more nearly coplanar with the plane defined by the four porphyrin nitrogens than in the high-spin Fe^{3+} case (Hoard *et al.*, 1965). The O–H (b) represents a hydrogen bond to an iron-bound hydroxyl oxygen; in this sense the ligand to iron is a protonated hydroxyl rather than a water molecule. Changes have been found in magnetic susceptibilities and electronic spectra of hemin methoxides with changes in solvent (low-spin and high-spin states predominate in pyridine and in chloroform, respectively) (B. D. McLees, G. T. Gregg, and W. S. Caughey, 1963, unpublished). Also loss of a sulfate in the vicinity of the N_δ of imidazole E7 on azide ion binding of metmyoglobin has been reported (Stryer *et al.*, 1964).

The narrowly split, asymmetric absorption between the wide pair of lines in the 298°K spectrum, signifying a small amount of high-spin iron, is probably due to hemin either free, or partially bonded to a denatured portion of the protein, or to a residual fraction of metmyoglobin from which the water had not been completely removed. However, attempts to further dry a methemoglobin sample by desiccation over P_2O_5 for

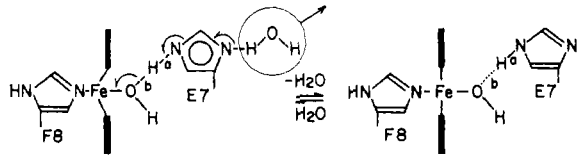

FIGURE 6: A model for the stabilization of the low-spin state of iron in metmyoglobin on dehydration.

48 hr did not reduce the intensity of a similar narrowly split fraction of the absorption.

Further Mössbauer work with suitable model compounds may be able to indicate which of the alternative explanations for stabilizing the low-spin state is the correct one. Mössbauer spectroscopy is uniquely adapted to settling this kind of question because the absorption is specific to the iron and the lines can be resolved with an accuracy sufficient to detect small changes in the ligand environment influencing nuclear energy levels.

In addition to the evidence for low-spin iron, some additional conclusions can be drawn from the Mössbauer spectrum. The observed isomer shift for the outer pair of lines indicates the amount of delocalization of the iron electrons. As was pointed out, the isomer shift is only affected directly by the s-electron density, but d-shell electron density has a strong indirect effect insofar as it shields the nucleus from the s electrons. In this sense the Mössbauer isomer shift indicated the true valence state iron, the actual electron density in the vicinity of the iron nucleus, as opposed to formal valence state. In the formal Fe^{2+}-dipyridine heme compounds, Mössbauer spectroscopy indicates that the effective charge or true valence state is nearly the same as in the formal-valence Fe^{3+} compounds (Bearden *et al.*, 1965b). This phenomenon is in agreement with molecular orbital calculations for low-spin Fe^{2+} compounds which predict a significant amount of electron delocalization to the ligand π orbitals (Shulman and Sugano, 1965).

For the outer pair of lines in the metmyoglobin spectra the isomer shift is slightly more negative (−0.2 mm/sec) than is observed for protohemin chloride (+0.08 mm/sec), indicating greater s-electron density at the iron nucleus. This can be due to less d-shell shielding since low-spin Fe^{3+} can act as a better π donor to surrounding ligands than can high-spin Fe^{3+}. A more negative (relative to the ^{57}Co:Cu source) isomer shift thus indicates a more covalent character for the low-spin iron bonding in lyophilized metmyoglobin as compared to the case of protohemin chloride. "More covalent" can be taken in the sense that the iron acts as a stronger acceptor of ligand σ electrons of sp character as well as to involve π bonding. However in a quantitative sense bonding to the surrounding ligands does not seem to be comparable to that observed for the formal-valence Fe^{2+} low-spin compounds (Bearden *et al.*, 1965b).

Attempts (Maling and Weissbluth, 1964) to correlate the isomer shift of the outer Mössbauer lines of lyophilized metmyoglobin or hemoglobin with the per cent 4s character of the predominantly d^5-electron configuration using the calculations of Walker *et al.* (1961) are not valid. These calculations were made using Hartree–Fock results for high-spin Fe^{3+}. The outer Mössbauer lines are due to low-spin Fe^{3+}, with very different ground-state wave functions.

The Mössbauer lines are plainly visible even at 298°K. This indicates that the probability of resonant absorption (*f* value) is substantial, and not highly temperature dependent. Kagan (1961) has pointed out that in polyatomic systems optical modes of vibration, where the atoms of a unit cell oscillate opposite to one another, become important. Myoglobin is such a polyatomic system, of course, and the strong resonant absorption we have observed is in full qualitative agreement with Kagan's work.

Thus far, the discussion has centered on the 228°K spectrum (Figure 2) although the temperature dependence of the various spectral features is obvious. Temperature-dependent line broadening due to unresolved magnetic hyperfine splitting has been discussed. In high-spin iron, an effective magnetic field (Fermi contact interaction) at the nucleus can arise from unpaired s-electron density at that position (Watson and Freeman, 1961). Even when all s electrons are paired, the density of spin-up electrons at the nucleus can differ due to differential shielding by the unpaired d-shell electrons. In Fe^{3+}, the effective magnetic field at the nucleus will be proportional to S_z of the d-shell electrons. Such a magnetic field can then split the nuclear $M_I = \pm 1/2$ state and cause an apparent broadening of the $M_I = \pm 3/2$ absorption line. Protohemin chloride displays Mössbauer spectra which are accounted for by this hypothesis (Bearden *et al.*, 1965b). The broadening disappears at 4.6°K because the low lying $S_z = \pm 1/2$ state becomes exclusively populated at the expense of the $S_z = \pm 5/2, \pm 3/2$ states. Since the effective magnetic field at the nucleus is proportional to S_z, the $S_z = \pm 1/2$ state causes much less broadening than the $S_z = \pm 3/2, \pm 5/2$ states. In the metmyoglobin spectra a similar situation occurs for the inner pair (high-spin iron) of lines (see Figures 1–3). Above 40°K the right-hand member of the inner pair (marked D in Figures 1–3) is broadened relative to the left-hand member. At 4.6°K the inner lines have equal widths and form a symmetric pair (Figure 4). This similarity in spectral behavior to protohemin chloride strengthens the identification of the inner lines with high-spin iron.

Neither member of the outer pair of lines appears to be broadened at 298 or 228°K (Figures 1 and 2). However, at 78°K the right-hand member of the wide pair is broadened though the areas of the two lines remain the same. Equality of these areas implies that the magnetic

[2] The unit 1 mm/sec = 11.6; Mc = 4.8×10^{-8}; ev = 3.8×10^{-4} cm^{-1}; this uncommon energy unit follows from a calculation of the energy shift in the 14.4-kev radiation due to a first-order relativistic Doppler shift.

hyperfine interaction is again responsible for the line broadening.[3] Since neither line is broadened above 78°K, it appears that at higher temperatures the relaxation time of the magnetic field direction at the nuclear position is much shorter than the precession period h/E_H of the nuclear moment. In general the low-spin ($S = {}^1/_2$) iron with $L \neq 0$ would be expected to have a shorter electron spin-lattice relaxation time at a given temperature than the $S = {}^5/_2, L = 0$ iron configuration (Wertheim, 1964). This fact is thus reflected in the observation that the inner pair of lines in the metmyoglobin spectra is broadened even at 228°K while the outer pair is only broadened at 78°K and below.

At 4.6°K, the wide pair of lines appears to be strongly reduced in intensity but there is a diffuse absorption on the order of 1% or less over a velocity range of ±6mm/sec (Figure 5). In the intermediate case when spin-lattice relaxation time is neither much longer nor much shorter than the nuclear precession period, broad, unresolved absorption can occur (Blume, 1965). At a sufficiently lower temperature, one would expect the spin-lattice relaxation time to become long enough to allow the resolution of well-defined hyperfine lines. However, Mössbauer spectra taken at 2.5°K still display diffuse absorption and do not show a resolved hyperfine pattern.

It is important to note that the temperature dependence of the spectrum is best explained by physical effects, rather than chemical changes. The chemical interpretation of the 228°K spectrum in terms of iron coordination is valid over the entire temperature range.

Appendix

Several factors contribute to the desirability of the use of ^{57}Fe-enriched samples for Mössbauer spectroscopy of iron proteins. These are: sample size conditions, the influence of scattered radiation, and the spectrometer time required per run.

In order to illustrate the advantages of using enriched samples, consider a typical Mössbauer cuvet of 5-cm² cross-sectional area. The 20 mg/cm² sample (mol wt 17,000) contains a single Fe atom per molecule or 6.8×10^{-5} g/cm² of iron. One resonant-recoilless radiation length (the absorber thickness which transmits 1/e of the incident radiation) for ^{57}Fe is 6.3×10^{-5} g/cm² of ^{57}Fe, or of the same order as the total Fe thickness in metmyoglobin. Therefore, a highly enriched (50–90%) metmyoglobin sample provides a reasonable portion of a resonant recoilless radiation length depending on the recoilless fraction of the particular absorber (~0.2 for metmyoglobin at 78°K).

An unenriched metmyoglobin sample would be 46.6 times thicker than a fully enriched sample as the natural abundance of ^{57}Fe is 2.19%. The thicker unenriched sample introduces considerable Compton and Thomson scattered radiation into the detector, particularly scattered 122-kev radiation from the ^{57}Co source. Photoelectric absorption of 14.4-kev radiation in unenriched metmyoglobin further lengthens the running time necessary to achieve adequate counting statistics in the Mössbauer spectra.

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[3] The Gol'danski effect predicts a difference in area between the members of a quadrupole pair under certain conditions. However, the lines should have the same width unless a variety of nuclear environments is present. Our spectra lines have *ca.* equal areas but very different widths. This is more compatible with broadening due to magnetic hyperfine splitting. For low-spin Fe^{3+} iron there will be a magnetic field at the nucleus due to the orbital moment of the electrons as well as the Fermi contact interaction.

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Additional Observations on the Chemistry of Clostridial Ferredoxin*

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ABSTRACT: Apoferredoxins were prepared from clostridial ferredoxin by three different methods: treatment with the mercurial, sodium mersalyl at pH 7.4, and treatment with the iron chelating agent, α,α'-dipyridyl, at both pH 5.4 and 7.4. The apoferredoxin samples showed no enzymatic activity and were free of iron and labile sulfide. Their amino acid analyses and total half-cystine contents were identical. None of the apoferredoxins could be reconverted to ferredoxin by the addition of ferrous iron. A quantitative and simultaneous release of both iron and sulfide occurs when ferredoxin is treated with α,α'-dipyridyl in the presence as well as in the absence of sodium hydrosulfite at pH 5.4. Only a slow release occurs in the absence of the iron chelating agent when hydrosulfite is present. No H_2S is released from oxidized ferredoxin in the absence of α,α'-dipyridyl during the 3-hr period of these experiments. If the evolved H_2S originated from cysteine residues in ferredoxin, a quantitative conversion of cysteine to dehydroalanine should occur. No significant amounts of dehydroalanine were detected in the samples of apoferredoxin examined. The model compounds, L-cysteine methyl ester hydrochloride and reduced glutathione, were tested for the evolution of H_2S under a variety of conditions, but no H_2S was evolved under any of these conditions. These results indicate that the inorganic sulfide of clostridial ferredoxin does not arise from the cysteine residues of the peptide chain.

The nature of the iron linkage and the origin of inorganic or labile sulfide in bacterial ferredoxin have not yet been determined. Previous work from this laboratory (Lovenberg *et al.*, 1963) demonstrated that all clostridial ferredoxins examined contain equivalent amounts of iron, inorganic sulfide, and cysteine residues. A close association of iron and inorganic sulfide in the native protein was indicated since removal of one of the two components was accompanied by the simultaneous loss of the second component.

A recent report by Bayer *et al.* (1965) postulated a structure for clostridial ferredoxin in which the iron is bound to the protein exclusively through the sulfur atoms of the cysteine residues in the peptide chain. The release of inorganic sulfide is postulated to occur through a β-elimination reaction from the cysteine residues. The main evidence supporting this model was the reported reconstitution of ferredoxin by the addition of ferrous ammonium sulfate alone to an apoferredoxin prepared by treatment of reduced ferredoxin with α,α'-dipyridyl. No source of inorganic sulfide was required for reconstitution of ferredoxin from this material.

The purpose of this paper is to report our investigations on the model proposed by Bayer *et al.* (1965). An examination of the properties of apoferredoxins prepared by three methods has been made and we have attempted to evaluate the model from these data. Evidence is presented which indicates that the H_2S evolved from native ferredoxin does not arise from the cysteine residues in the protein.

Experimental Procedures

Materials. The following reagents were purchased from commercial sources: L-cysteine methyl ester hydrochloride, reduced glutathione and sodium *o*-[(3-hydroxymercuri-2-methoxypropyl)carbamyl]phenoxy-

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