MÖSSBAUER SPECTROSCOPIC STUDIES OF HEMOGLOBIN AND ITS ISOLATED SUBUNITS

G. R. HOY,* D. C. COOK,* R. L. BERGER,‡ AND F. K. FRIEDMAN§
*Physics Department, Old Dominion University, Norfolk, Virginia 23508; ‡Laboratory of Technical Development, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892; and §Laboratory of Molecular Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

ABSTRACT Samples of 90% enriched ^{57}Fe hemoglobin and its isolated subunits have been prepared. Mössbauer spectroscopic measurements have been made on three such samples. Sample one contained contributions of oxyhemoglobin, deoxyhemoglobin, and carbonmonoxyhemoglobin. This sample was studied from a temperature of 90 K down to 230 mK. Measurements were also made at 4.2 K using a small applied magnetic field of 1.0 T. In general, the measured quadrupole splittings and isomer shifts for each component agreed with previous measurements on single component samples in the literature, and thus demonstrated that chemically enriched hemoglobin has not been altered. The second and third samples were isolated α and β subunits, respectively. We have found measurable Mössbauer spectral differences between the HbO_2 sites in the α subunit sample and the β subunit sample. The measured Mössbauer spectral areas indicate that the iron ion has the largest mean-square displacement at the deoxy Hb sites as compared to that at the oxy- and carbonmonoxy Hb sites. The mean-square displacement at the HbO_2 sites is the smallest.

INTRODUCTION

Hemoglobin has been the subject of numerous investigations for many years because of its vital function as an oxygen carrier in vertebrates. However, the processes that govern the reversible binding of O_2 to iron in the heme pockets is still something of a mystery. Pauling and Coryell (1) argued, based on susceptibility measurements, that before oxygen binding, the deoxyhemoglobin system exists in which the iron ion in the heme is in a high-spin, ferrous state. When the O_2 oxygen molecule becomes bound to the iron, the state changes to a diamagnetic low-spin ferrous configuration characteristic of the oxyhemoglobin system. Mössbauer spectroscopy is a particularly useful technique for the study of systems such as oxyhemoglobin, deoxyhemoglobin, and carbonmonoxyhemoglobin because of the method's sensitivity to charge and spin distributions of electrons in the neighborhood of the iron site. The first Mössbauer effect experiments were done in the early 1960's (2–5). Such studies reached maturity with the work of Lang and Marshall (6). The general conclusions of this earlier research were that the iron ion in oxyhemoglobin and in carbonmonoxyhemoglobin is in the low-spin ferrous configuration ($S = 0$), in deoxyhemoglobin it is in the high-spin ferrous configuration ($S = 2$).

There has been considerable progress made in understanding the Mössbauer results in oxyhemoglobin (6), including a number of theoretical investigations (7–13). Magnetic susceptibility measurements on oxyhemoglobin (14–18) have also supported the observation that oxy- and carbonmonoxyhemoglobin are both low spin. Recently, Tsai et al. (19) have proposed an interesting model for the iron dioxygen bond in HbO_2.

For many years, Mössbauer spectroscopy has been applied to solid state physics problems in which the sample under study had several different iron components. This has not usually been the case in biophysics applications, because of concern about the biochemical integrity of the sample, as well as the lack of knowledge of the required Mössbauer parameters. The extensive Mössbauer spectroscopic data concerning hemoglobin now allow investigations of mixed samples. The study of mixed samples can be very useful when one wishes to make a direct comparison between the components. This is true because the external parameters, e.g. applied magnetic field, and background effects are identical for each component. Here, we present studies of an ^{57}Fe enriched sample of hemoglobin consisting of a mixture of the three components mentioned above. Thus we can, for example, directly compare the high-spin or low-spin structure of Fe^{II} in HbO_2, HbCO, and deoxy Hb by using an external magnetic field. In addition to this sample, we also have results for the isolated α and β subunits. Mössbauer effect measurements were made at temperatures from 135 K down to 70 mK with and without an externally applied magnetic field. The extremely low temperature measurements were done to determine if any additional hyperfine structure appeared.

EXPERIMENTAL PROCEDURES AND SAMPLE PREPARATION

Mössbauer spectra were recorded using a 25 mCi source of ^{57}Co in a rhodium matrix. The electromechanical drive is of conventional design, and the source was driven in the constant acceleration mode. The usual transmission geometry was used in which the gamma rays from the source, inside the cryostat, go through the absorber and are detected by a commercially made Xe-CO_2 proportional counter. The velocity calibration was established by using a standard 0.001 inch thick natural iron foil absorber with both source and absorber at 300 K. Line widths of 0.25 ± 0.01 mms^{-1} were obtained in the calibration spectra. No attempt was made to maintain the source at room temperature when the samples were cooled down. The hemoglobin and subunit samples were kept at liquid N_2 temperatures when not in use. When starting an experiment, the frozen sample was placed in the mixing chamber of our dilution refrigerator-Mössbauer apparatus that was at room temperature. The cryostat was then pumped down and cooled to liquid nitrogen temperature as quickly as possible. It took ~90 min to load the sample and reach a temperature of <250 K. During that period the sample was sealed in its specially designed airtight holder inside the mixing chamber. Once the sample was inserted in the apparatus, all measurements were made at the corresponding temperature before bringing the sample up to room temperature for its removal.

The dilution refrigerator-Mössbauer apparatus is capable of maintaining sample temperatures down to 70 mK. A magnetic field of 1.0 T can be applied to the sample while the source remains in a zero-field region. The applied magnetic field is parallel to the gamma ray direction. The temperature of the absorber, i.e. the sample, is monitored by a silicon diode sensor for temperatures above 2 K. Below this temperature a carbon resistor monitors the sample temperature down to 0.5 K. A cerium magnesium nitrate salt pill, as part of a magnetic susceptibility bridge, is used to monitor temperatures down to 70 mK. The accuracy and stability of our system for all temperatures at or below 4.2 K is ±5 mK.

Mössbauer spectra were recorded for the samples at several temperatures and at the lower temperatures with and without an applied magnetic field. Experimental runs lasted from 6 h (0.26×10^6 counts per channel) up to 2 d (2×10^6 counts per channel) depending on the sample used, and the holding time for the liquid helium at pumped helium temperatures. The resulting spectra were then fitted using a Lorentzian least-squares computer routine assuming a certain number of Lorentzian lines in each spectrum. The width, intensity, and location of each Lorentzian line were allowed to vary independently. Fitting the spectra in this fashion allowed us to examine the credibility of the parameters obtained. The intensities, as well as the areas, of each line in a quadrupole split (two-line) Mössbauer spectrum of a polycrystalline specimen should theoretically be equal. In a numerical fitting procedure such as we used, these criteria will not be perfectly satisfied. Numerical results depend on the quality of the data and the prominence of the particular lines in question. Consider Fig. 1, which shows Mössbauer spectra for sample one, which contains contributions from oxyhemoglobin, deoxyhemoglobin, and carbonmonoxyhemoglobin. To obtain an idea of the quality of the fitting procedure, we quote the criteria check given above for this case. The average numerical error in the inequality of the intensities of the two lines in the HbO_2 spectra is <5%, in the Hb(deoxy) spectra is 10%, and in the HbCO spectra is 0.5%. The average numerical error in the inequality of the areas of the two lines in the HbO_2 spectra is <2%, in the Hb(deoxy) spectra is 6%, and in the HbCO spectra is ~10%. The positions of the lines and hence the quadrupole splitting and isomer shift values (see Tables I, II, and III) agree with previous results. Additionally, one expects that the line widths of "thin" samples at low temperatures should approach the calibration spectra line widths given above. The values obtained for our line widths reasonably satisfy this criterion (see Tables IV, V, and VI). Such calculations and observations, together with the overall temperature dependence of the parameters and their internal consistency, provide some confidence in the resultant fits to the spectra.

Hemoglobin A was obtained from human erythrocytes and purified by ion exchange chromatography (20) using DEAE-Sepacel (Pharmacia Inc., Pisacataway, NJ). HbA was converted to methemoglobin with potassium ferricyanide and filtration through a Sephadex G-25 (Pharmacia) column. Apohemoglobin was prepared by the butanone method (21). For reconstitution, apohemoglobin was first dialyzed against 0.1 M potassium phosphate (pH 7.0). To reduce the labor involved in the enrichment, our earlier procedure (34) was modified. ^{57}Fe hemin (Porphyrin Products, Logan, Utah) (1.2 equivalents) was added at 0°C and the reaction mixture was dialyzed against 0.1 M Tris-HCl (pH 8.5). The ^{57}Fe Hb was purified by adsorption on a DEAE-Sephacel column with this buffer, and elution with 0.02 M potassium phosphate (pH 7.5). The reconstituted ^{57}Fe Hb was dialyzed against 0.1 M potassium phosphate (pH 7.0). It was then converted to the carbonmonoxy form by treatment with sodium dithionite and CO, passed through a Sephadex G-25 column to remove excess dithionite, and stored at −60°C. The α and β subunits were prepared by dissociation of ^{57}Fe-enriched hemoglobin with p-mercuribenzoate, separation of the subunits by ion-exchange chromatography, and regeneration of sulfahydryls, all according to standard procedures (22–24). The samples were analyzed using standard spectral photometric analysis in the visible region.

Approximately 0.7 cc of each sample at 0°C was placed in a specially designed thin walled Delrin container (Dupont, Wilmington, Delaware), which was kept cold with dry ice. A thin Delrin disc was then sealed over the sample by means of an O-ring. The samples were frozen within 2 min with dry ice. The concentration of ^{57}Fe in the first, second, and third samples were 10.4, 2.0, and 0.22 mM, respectively. These figures give Mössbauer absorber thicknesses of 0.47 mg cm^{-2}, 0.064 mg cm^{-2}, and 0.010 mg cm^{-2}, respectively.

RESULTS

Fig. 1 shows our experimental results for an ^{57}Fe-enriched hemoglobin sample. The solid lines are Lorentzian least-squares fits to the data allowing the position, width, and the intensity of the lines to vary independently. The spectra were fitted assuming eight lines in spectrum *a* and six lines in spectra *b* and *d*. The experimental spectra arise from sets of quadruple split spectra corresponding to oxyhemoglobin, deoxyhemoglobin, and carbonmonoxyhemoglobin Mössbauer patterns, (see doublet positions in *a* and *b* of Fig. 1). The values of some of the derived parameters are collected in Table I.

Generally speaking, the values of the quadrupole splittings and isomer shifts as a function of temperature agree with those obtained by other investigators (6, 34). Notice that at 90 K the oxyhemoglobin pattern clearly has two components, *i* and *ii*. These are labeled $HbO_2(1)$ and $HbO_2(2)$ in Table I. This phenomenon has also been previously observed. A comparison of Fig. 1 *b* and *c* gives a clear indication about the possible iron ion electron configurations at the iron sites for the three components of these spectra. As mentioned above, the ferrous iron ion is expected to be in the singlet $S = 0$ state for both the oxy- and carbonmonoxyhemoglobin sites. The iron in this state is diamagnetic, and hence the effective magnetic field at the iron nucleus at these sites should be given essentially by the applied magnetic field. In fact, the measured line broadening in Fig. 1 *c* at the oxy- and carbonmonoxyhemoglobin sites does correspond to 1.0 T. However, the ferrous iron ions at the deoxyhemoglobin sites are expected to be in an electron configuration, $S = 2$, high-spin state. As such, even a small, externally applied magnetic field should

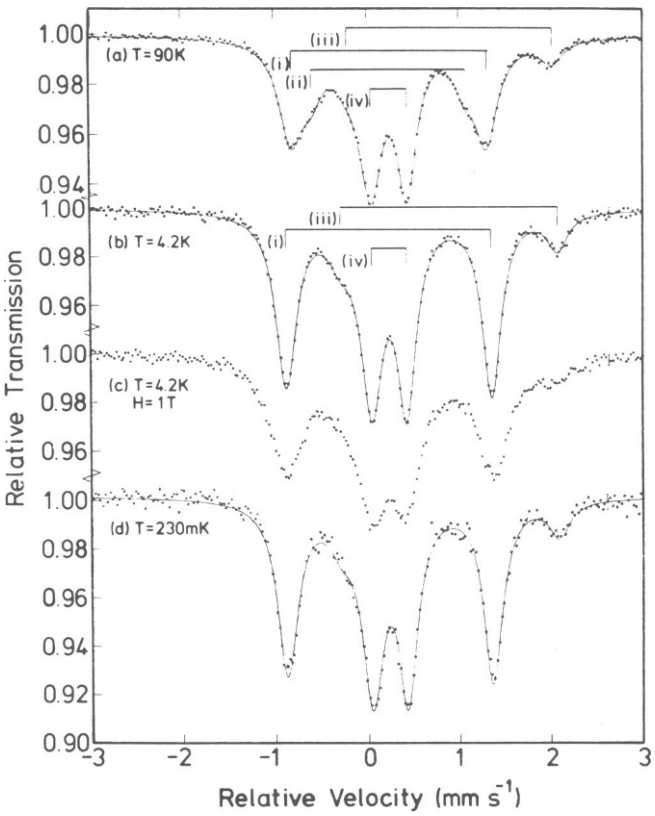

FIGURE 1 Mössbauer spectra of the enriched Hb sample recorded at (*a*) 90 K, (*b*) 4.2 K, (*c*) 4.2 K in an applied magnetic field of 1 T, and (*d*) 230 mK. The spectra are composed of sets of quadrupole doublets (*i*) $HbO_2(1)$, (*ii*) $HbO_2(2)$, (*iii*) Hb(deoxy), and (*iv*) HbCO as indicated on the upper half of the figure.

induce a rather large effective magnetic field due to core polarization, excluding an ionic $S_Z = 0$ ground state, which would result in an effective magnetic field at the iron nucleus corresponding to ~20 T. This would result in a very smeared out spectrum and not just a line broadening as seen at the oxy- and carbonmonoxyhemoglobin sites. Notice that the dip on the right side of Fig. 1 *b* corresponding to one of the quadrupole split lines in the deoxyhemoglobin spectrum is smeared out by the externally applied weak magnetic field (Fig. 1 *c*). This direct comparison reconfirms the Fe^{II} spin states in these components at 4 K.

Fig. 2 shows similar results for the sample of separated ^{57}Fe-enriched α subunits. Again we observe the quadrupole split doublet pattern corresponding to the same oxyhemoglobin sites (Fig. 2 *a*) at 135 K. Also, comparing Fig. 2 *b* and *c*, we see again that the ferrous ions at the deoxyhemoglobin sites are not in a low-spin configuration as is the case for the ferrous ions at the oxyhemoglobin sites. Notice further that the carbon monoxyhemoglobin contribution has been reduced greatly owing to partial dissociation of the CO ligand from the heme during the procedures in which the ^{57}Fe-enriched α subunit was prepared from ^{57}Fe-enriched Hb. Table II summarizes some of the parameters determined for the ^{57}Fe α chain sample.

TABLE I
SOME PARAMETERS DETERMINED FROM A LEAST-SQUARES FITTING OF THE MÖSSBAUER SPECTRA OF THE ENRICHED Hb SAMPLE SHOWN IN FIG. 1

	Sub-spectrum	Contribution	Quadrupole splitting, ΔE_Q	Isomer shift,* δ	Temperature
		%	mms^{-1}	mms^{-1}	
(i)	HbO_2 (1)	33(1)‡	2.12(1)	0.23(1)	90K
(ii)	HbO_2 (2)	13(1)	1.68(1)	0.23(1)	90K
(iii)	Hb (deoxy)	9(1)	2.23(2)	0.89(2)	90K
(iv)	HbCO	45(1)	0.39(1)	0.23(1)	90K
(i)	HbO_2 (i)	43(1)	2.24(1)	0.23(1)§	4.2K
(ii)	HbO_2 (2)	0	—	—	4.2K
(iii)	Hb (deoxy)	9(1)	2.37(2)	0.88(2)	4.2K
(iv)	HbCO	48(1)	0.38(1)	0.22(1)	4.2K
(i)	HbO_2 (1)	43(1)	2.24(1)	0.23(1)	230 mK
(ii)	HbO_2 (2)	0	—	—	230mK
(iii)	Hb (deoxy)	9(1)	2.38(2)	0.89(2)	230mK
(iv)	HbCO	48(1)	0.38(1)	0.22(1)	230mK

*The isomer shift is measured relative to a 0.001" natural iron foil absorber at room temperature.
‡The number in parentheses indicates the error in the last significant figure.
§The low temperature values of δ were not corrected for the fact that the source temperature was not held fixed.

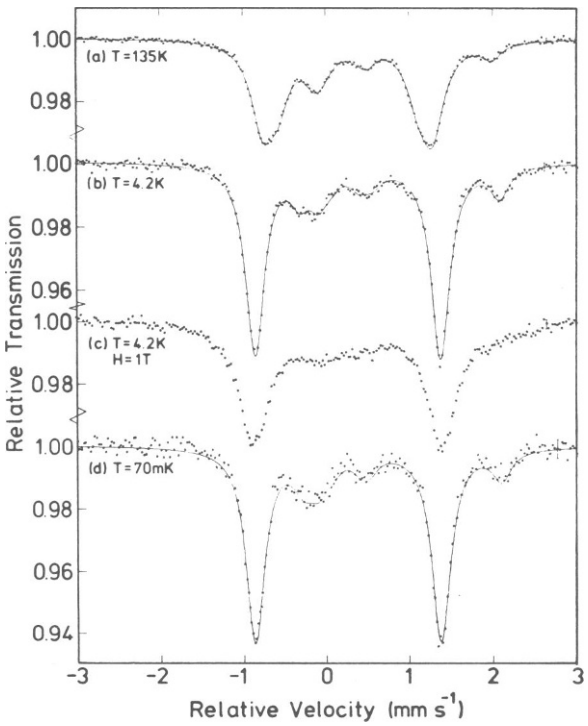

FIGURE 2 Mössbauer spectra of the enriched α subunit sample recorded at (*a*) 135 K, (*b*) 4.2 K, (*c*) 4.2 K in an applied magnetic field of 1 T and (*d*) 70 mK.

TABLE II.
SOME PARAMETERS DETERMINED FROM A LEAST-SQUARES FITTING OF THE MÖSSBAUER SPECTRA OF THE SEPARATED ^{57}Fe ENRICHED α SUBUNIT SAMPLE SHOWN IN FIG. 2.

	Sub-spectrum	Contribution	Quadrupole splitting, ΔE_Q	Isomer shift, δ	Temperature
		%	mms^{-1}	mms^{-1}	
(i)	HbO_2 (1)	50(1)	2.05(1)	0.24(1)	135K
(ii)	HbO_2 (2)	28(1)	1.69(1)	0.25(1)	135K
(iii)	Hb (deoxy)	8(1)	2.17(2)	0.88(2)	135K
(iv)	HbCO	5(1)	0.37(2)	0.21(2)	135K
(v)	impurity	9(1)	—	—	135K
(i)	HbO_2 (1)	74(1)	2.24(1)	0.23(1)	4.2K
(ii)	HbO_2 (2)	0	—	—	4.2K
(iii)	Hb (deoxy)	11(1)	2.38(2)	0.87(2)	4.2K
(iv)	HbCO	6(1)	0.37(2)	0.21(2)	4.2K
(v)	impurity	9(1)	—	—	4.2K
(i)	HbO_2 (1)	74(1)	2.24(1)	0.23(1)	70mK
(ii)	HbO_2 (2)	0	—	—	70mK
(iii)	Hb (deoxy)	12(1)	2.38(2)	0.89(2)	70mK
(iv)	HbCO	5(1)	0.38(2)	0.21(2)	70mK
(v)	impurity	9(1)	—	—	70mK

Fig. 3 shows our results for the ^{57}Fe-enriched β chain subunit sample. In this case, the sample preparation procedure for the ^{57}Fe-enriched β chains resulted in a significant reduction in the carbonmonoxyhemoglobin contribution and completely eliminated the deoxyhemoglobin contribution. The quadrupole doublet Mössbauer pattern corresponding to the oxyhemoglobin sites at higher temperatures, (90 K), is still present in the enriched β chain sample. Thus, this feature is seen in all three samples. Some of the characteristic parameters obtained by analysis of our data (Fig. 3) on the enriched β chain sample are given in Table III.

Figs. 2 and 3 indicate the presence of an impurity in each of the samples. These impurity spectra were fitted in each case with two lines whose separation is 0.65 ± 0.02 mms^{-1}. The left peak, situated at −0.13 ± 0.02 mms^{-1} with respect to the iron calibration spectrum, is twice as intense as the right peak. The isomer shift corresponding to the pair is +0.20 ± 0.02 mms^{-1}. Due to the narrow line width, 0.22 ± 0.03 mms^{-1}, of the larger peak at 90 K in sample 3, (Fig. 3 *a*), we suspect the iron ion in the impurity is undergoing spin relaxation. Problems of hemochrome formation are known to arise in the preparation of isolated subunit samples (25).

A plot of the quadrupole splittings for all of our subspectra in each sample as a function of temperature is given in Fig. 4. The top section of Fig. 4 shows our results for the oxyhemoglobin sites in all samples. All samples showed the presence of two subspectra for the HbO_2 sites at temperatures of 90 K and above. The minority spectrum, corresponding to the smaller electric field gradient, appears to have a constant electric field gradient at the iron

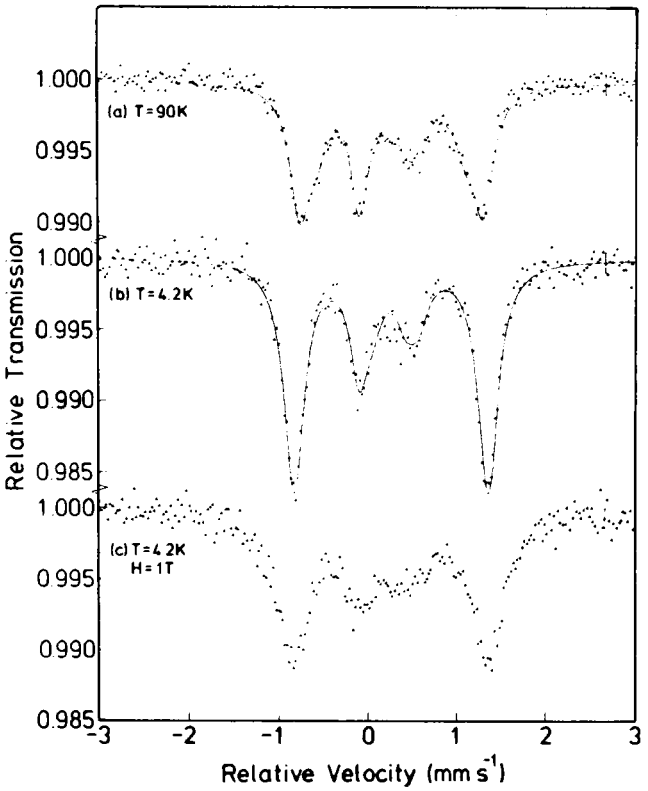

FIGURE 3 Mössbauer spectra of the enriched β subunit sample recorded at (*a*) 90 K, (*b*) 4.2 K, and (*c*) 4.2 K in an applied magnetic field of 1 T.

site in all three samples at temperatures of 90 K and 135 K. Ignoring this minority site, the temperature dependence of the quadrupole splittings at the HbO_2 sites corresponds to that observed by other investigators. However, although the quadrupole splittings at the HbO_2(1) site for the hemoglobin and α subunit samples are the same, those in the β subunit sample are decidedly different.

TABLE III
SOME PARAMETERS DETERMINED FROM A LEAST-SQUARES FITTING OF THE MÖSSBAUER SPECTRA OF THE SEPARATED ^{57}Fe ENRICHED β SUBUNIT SAMPLE SHOWN IN FIG. 3

	Sub-spectrum	Contribution	Quadrupole splitting, ΔE_Q	Isomer shift, δ	Temperature
		%	mms^{-1}	mms^{-1}	K
(i)	HbO_2 (1)	41(1)	2.06(2)	0.24(2)	90
(ii)	HbO_2 (2)	21(1)	1.69(2)	0.23(2)	90
(iii)	Hb (deoxy)	0	—	—	90
(iv)	HbCO	12(1)	0.36(2)	0.20(2)	90
(v)	impurity	26(1)	—	—	90
(i)	HbO_2 (1)	67(1)	2.18(2)	0.24(2)	4.2
(ii)	HbO_2 (2)	0	—	—	4.2
(iii)	Hb (deoxy)	0	—	—	4.2
(iv)	HbCO	12(1)	0.38(2)	0.21(2)	4.2
(v)	impurity	21(1)	—	—	4.2

The middle section of Fig. 4 shows our results for the quadrupole splittings at the deoxyhemoglobin sites in the hemoglobin and α subunit samples. The quadrupole splittings and their temperature dependences are the same in both samples.

The bottom section in Fig. 4 shows our results for the quadrupole splittings at the HbCO sites in all three samples. The quadrupole splittings are temperature and sample independent within the experimental errors.

Since our spectra, particularly the enriched hemoglobin sample, show rather clearly the HbO_2, deoxyhemoglobin, and HbCO components, it is possible to investigate the mean square displacement of the iron atoms in these components. This involves the concept of the recoilless fraction and its relationship to the measured area in a Mössbauer spectrum (Figs. 1, 2, and 3) of each subspectrum. Since each subspectrum corresponds to a particular component in the sample, a comparison of the mean square displacement of the iron ions in each component is possible because the background effects are the same.

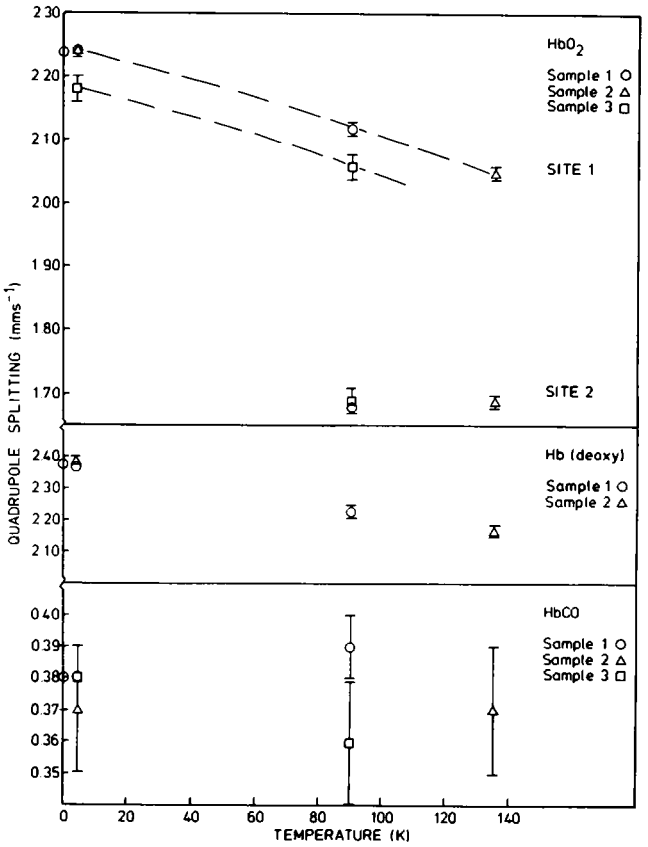

FIGURE 4 The measured quadrupole splittings as a function of temperature for the three major components in our three samples. The top section shows our results for HbO_2 in all three samples including sites 1 and 2. The temperature dependence of the site 1 component agrees with previous results. However, the magnitudes of the quadrupole splittings in sample 3 (β subunit sample) are clearly smaller than those measured in samples 1 (Hb sample) and 2 (α subunit sample). The measured quadrupole splittings for site 2 appear to be temperature independent between 90 K and 135 K. The middle section of the figure illustrates the measured quadrupole splittings for the Hb(deoxy) component. The trend and values also agree with previous published data. The bottom section shows the measured quadrupole splittings for the HbCO component to be approximately temperature independent.

The relationship between the experimentally determined Mössbauer spectrum area and the recoilless fraction (f), which is related to the mean square displacement of the iron atom, has been discussed in detail (26, 27). Since proteins are well recognized as fluctuating systems, the influence of protein dynamics on Mössbauer spectra has, in recent years, become a very interesting field of research (28). Application of such ideas to protein structure dynamics has been used in studying metmyoglobin (29), deoxymyoglobin (30), deoxyhemoglobin, and hemochrome (31). To obtain an estimate of the relative f values from our data, we have assumed that the proportionality constant between f factors and the absorption area is given by setting $f = 0.8$ at 4.2 K. This approach has been used previously (31). Therefore we can write,

$$\ln f_i = -\frac{\langle x^2(T)\rangle_i}{\lambda\!\!\!^{-2}} \propto \text{Area}^{(i)}_{\text{observed}}, \qquad (1)$$

where $\langle x^2(T)\rangle_i$ is the mean square displacement of the iron atom at the ith site when the sample temperature T is in Kelvin, and $\lambda\!\!\!^{-}$ is the reduced wavelength of the gamma radiation emitted by the source. In this case $\lambda\!\!\!^{-} \cong 0.14$ Å.

Tables IV, V, and VI show the calculated results for the mean-square displacements per $\lambda\!\!\!^{-}$ for iron at the HbO_2, Hb(deoxy), and HbCO sites in the hemoglobin sample and in the α subunit sample, and at the HbO_2 and HbCO sites in the β subunit sample.

A plot of the iron atoms, mean-square displacements at the various sites as a function of temperature taken from Tables IV, V, and VI, is shown in Fig. 5. It is of interest to note that the mean square displacements of the iron atom is

TABLE IV
PARAMETERS USED TO DETERMINE THE IRON ATOMS' MEAN SQUARE DISPLACEMENTS IN THE ENRICHED Hb SAMPLE. THE LINEWIDTHS AND AREAS OF EACH SUBSPECTRUM HAVE BEEN DETERMINED USING A LEAST-SQUARES FITTING OF THE SPECTRA SHOWN IN FIG. 1

	Sub-spectrum	Line-width, Γ	Area of Sub-spectrum	Tempera-ture	Recoilless Fraction f	$\left\langle \frac{x^2(T)}{\lambda\!\!\!^{-2}} \right\rangle$
		mms^{-1}				
(i)	HbO_2(1)	0.30(1)	1.358(14)	90K	0.68(2)	0.39(3)
(ii)	HbO_2(2)	0.29(1)	0.531(5)	90K		
(iii)	Hb(deoxy)	0.33(1)	0.354(14)	90K	0.58(3)	0.54(5)
(iv)	HbCO	0.27(1)	1.869(18)	90K	0.61(2)	0.49(3)
(i)	HbO_2(1)	0.26(1)	2.225(22)	4.2K	0.80(1)	0.22(1)
(iii)	Hb(deoxy)	0.29(1)	0.490(20)	4.2K	0.80(3)	0.22(3)
(iv)	HbCO	0.26(1)	2.444(24)	4.2K	0.80(1)	0.22(1)
(i)	HbO_2(1)	0.26(1)	2.213(22)	230mK	0.80(1)	0.22(1)
(iii)	Hb(deoxy)	0.30(1)	0.476(19)	230mK	0.78(4)	0.25(5)
(iv)	HbCO	0.27(1)	2.442(24)	230mK	0.80(2)	0.22(2)

TABLE V

PARAMETERS USED TO DETERMINE THE IRON ATOMS' MEAN SQUARE DISPLACEMENTS IN THE α SUBUNIT SAMPLE. THE LINEWIDTHS AND AREA OF EACH SUBSPECTRUM HAVE BEEN DETERMINED USING A LEAST-SQUARES FITTING OF THE SPECTRA SHOWN IN FIG. 2

Sub-spectrum	Line-width, Γ	Area of Sub-spectrum	Tempera-ture	Recoilless Fraction f	$\left\langle \frac{x^2(T)}{\lambda^2} \right\rangle$
	mms^{-1}				
HbO_2(1)	0.34(1)	1.035(10)	135K	0.66(1)	0.42(2)
HbO_2(2)	0.33(1)	0.581(6)	135K		
Hb(deoxy)	0.28(1)	0.163(6)	135K	0.42(3)	0.87(7)
HbCO	0.34(2)	0.098(6)	135K	0.54(5)	0.62(9)
HbO_2(1)	0.27(1)	1.952(20)	4.2K	0.80(1)	0.22(1)
Hb(deoxy)	0.27(1)	0.307(12)	4.2K	0.80(3)	0.22(3)
HbCO	0.28(2)	0.145(9)	4.2K	0.80(5)	0.22(6)
HbO_2(1)	0.27(1)	1.991(20)	70mK	0.82(1)	0.20(1)
Hb(deoxy)	0.30(1)	0.337(13)	70mK	0.88(3)	0.13(3)
HbCO	0.27(2)	0.145(9)	70mK	0.80(5)	0.22(6)

TABLE VI

PARAMETERS USED TO DETERMINE THE IRON ATOMS' MEAN SQUARE DISPLACEMENTS IN THE β SUBUNIT SAMPLE. THE LINEWIDTHS AND AREA OF EACH SUBSPECTRUM HAVE BEEN DETERMINED USING A LEAST-SQUARES FITTING OF THE SPECTRA SHOWN IN FIG. 3

Sub-spectrum	Line-width, Γ	Area of Sub-spectrum	Tempera-ture	Recoilless Fraction f	$\left\langle \frac{x^2(T)}{\lambda^2} \right\rangle$
	mms^{-1}		K		
HbO_2(1)	0.26(1)	0.247(5)	90	0.57(2)	0.56(3)
HbO_2(2)	0.32(1)	0.124(2)	90		
HbCO	0.43(4)	0.070(14)	90	0.60(15)	0.51(30)
HbO_2(1)	0.27(1)	0.518(10)	4.2	0.80(2)	0.22(2)
HbCO	0.28(3)	0.094(18)	4.2	0.80(15)	0.22(20)

largest at the deoxyhemoglobin sites, smaller at the carbonmonoxyhemoglobin sites, and smallest at the oxyhemoglobin sites.

The fitted line widths of the component subspectra for each sample are also given in Tables IV, V, and VI. Specifically, at 4.2 K the line width of the HbO_2 spectrum is 0.27 ± 0.01 mms^{-1}. This agrees with the results reported by Tsai et al. (19) for HbO_2 and the isolated α subunit sample. At higher temperatures the line widths generally increase probably due to relaxation effects. We also see this general trend in our other line width results.

DISCUSSION AND CONCLUSIONS

Samples of ^{57}Fe-enriched hemoglobin, α chain subunits, and β chain subunits have been prepared. The hemoglobin sample had HbO_2, Hb(deoxy), and HbCO components present. The α chain subunit sample had HbO_2, Hb(deoxy), HbCO, and an impurity. The β chain subunit sample had only an HbO_2 component together with a relatively large contribution of the impurity. The fact that the samples contained the three major well known components, (HbO_2, Hb(deoxy), and HbCO) allowed a direct comparison of the results. In general, our measured quadrupole splittings and isomer shifts agree with previous results. We have reconfirmed the Fe^{II} spin states in the three components at 4 K.

The asymmetric line shape of the oxyhemoglobin spectrum at elevated temperatures has been observed previously. This effect is most clearly seen in our data in spectrum *a* of Fig. 1. Similar "shoulders" also have appeared in other researchers' data (32). To explain such results one needs to postulate the existence of an iron ionic excited state or an inequivalent iron site in the HbO_2 molecule. The true origin of this effect requires additional research. If the explanation is due to an excited state, our data would estimate that it lies above the ground state by ~ 55 cm^{-1}.

In comparing the quadrupole splittings at the HbO_2 sites in the α and β subunit samples, we find that the α subunit gives the same result as that found in the hemoglobin sample. On the other hand, the quadrupole splittings found in the β subunit sample are smaller. This trend also has been recently observed by others (19), but as yet is unexplained.

The electric field gradient (EFG) at the iron atom in the HbCO sites is small, in agreement with the measurement of others, and as expected from an Fe^{II} low-spin, $S = 0$, electronic configuration. We find no temperature dependence of the EFG and the same value in all three samples within experimental error. Thus, there do not appear to be any major structural transitions in these samples from 70 mK up to 135 K.

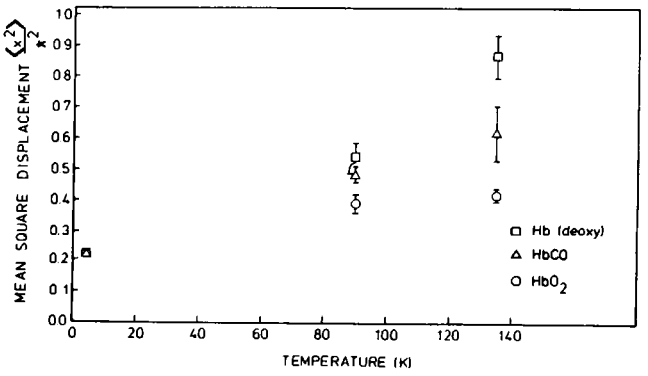

FIGURE 5 The mean square displacements of the iron atoms for each component show the expected increase with temperature. These results are calculated by using the measured areas for each component subspectrum as described in the text. Notice that the mean square displacements for the iron atoms at elevated temperatures in Hb(deoxy) are largest, in HbCO are less, and in HbO_2, the least.

Using the measured Mössbauer spectral areas (see Fig. 5), we conclude that the iron atoms at the deoxyhemoglobin site experience the largest mean square displacement while at the carbonmonoxyhemoglobin site it is smaller, and at the oxyhemoglobin site it is smallest. The measured root–mean-square displacements of the iron atoms at 90 K are ~0.27, 0.26, and 0.23 Å at the Hb(deoxy), HbCO, and HbO_2, sites, respectively. One might expect a trend of this sort from the simple observation that at the deoxy site the sixth ligand position for the iron is unoccupied, while in the carbonmonoxy and oxy forms, this position is occupied by successively larger molecules.

We authors wish to thank Professors George Lang and Hans Frauenfelder for helpful comments and criticism. We are also grateful to Dr. G. E. Copeland for help with the least-squares curve fitting routine. One of us, D. C. Cook, is appreciative of the summer support from Mr. R. H. Carpenter of Commonwealth Technology Incorporated.

The authors also wish to acknowledge partial support from the National Institutes of Health research grant #1-RO3-RR02178-01A1.

Received for publication 1985 and in final form 21 October 1985.

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