Collective Motion of Iron and Exogenous Ligands in Hemoglobin

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ABSTRACT: Mössbauer studies of deoxyhemoglobin (Hb), oxyhemoglobin (HbO_2), and (carbonmonoxy)-hemoglobin (HbCO) have been performed providing information regarding the center shift (CS) up to 250 K and the recoil-free fraction (f) in the "solid state regime". The temperature dependence of the CS indicates that the effective mass of the iron in liganded hemoglobin includes the exogenous ligand, suggesting that vibrations of the iron are strongly coupled to those of the ligand. As a result of this coupled motion, the recoil-free fraction (f) provides a valuable probe for the shape of the potential energy surface in the ligand pocket. Thus HbO_2 experiences a stronger restoring force than HbCO, which can be attributed to a steeper potential well for the oxygen into the ligand pocket.

In recent years, the use of the Mössbauer effect for the study of heme protein dynamics has been demonstrated (Parak & Reinisch, 1986; Mayo et al., 1981; Parak et al., 1981; Parak & Knapp, 1984; Keller & Debrunner, 1980; Levy et al., 1984). However, these studies have primarily utilized the recoil-free fraction (f) and have for the most part neglected the temperature dependence of the center shift (CS), which provides complementary dynamic information. Furthermore, the main thrust of the Mössbauer recoil-free fraction studies were directed at the temperature regime above the critical temperature (T_{cr}) at which the f factor departs from the expected behavior of a solid. These studies at elevated temperatures (above T_{cr}) have provided valuable information about the nature of motion at the iron site.

In this work, a comparative study of three hemoglobin complexes, deoxyhemoglobin (Hb), oxyhemoglobin (HbO_2), and (carbonmonoxy)hemoglobin (HbCO), utilizing both the temperature dependence of the CS up to 250 K and the f factor in the "solid state regime" (below T_{cr}) is reported. The results indicate a rigid lattice at the iron center in HbO_2, while an appreciable softening of the lattice is observed in the case of HbCO. Moreover, the effective mass of the iron in liganded hemoglobin suggests a mode of vibration in which vibrations of the iron are strongly coupled to those of the ligand.

Experimental Procedures

^{57}Fe-enriched horse hemoglobin was prepared as methemoglobin by Porphyrin Products (Logan, UT), according to the procedures described by Ascoli et al. (1981). A comparison of the ^{57}Fe-reconstituted and native methemoglobin was made by isoelectric focusing as well as visible and electon spin resonances spectroscopy. ^{57}Fe-reduced hemoglobin was prepared from methemoglobin in a Nesbitt bubbler by dithionite reduction under nitrogen. Excess dithionite was removed by subsequent dialysis in the same bubbler, prior to exposure of the samples to oxygen or carbon monoxide. In this way, potential damage caused by the reaction products of oxygen and dithionite was minimized.

The samples for Mössbauer measurements were transferred to a Lucite holder that provides a disk-shaped space of 0.4 cm in thickness and 1.25 cm in diameter. For deoxyhemoglobin measurements, this transfer was performed in a nitrogen atmosphere and the sample immediately frozen. Transmission Mössbauer spectra were obtained on a 512-channel spectrometer operated in a constant acceleration mode. A 100 mCi source of ^{57}Co diffused into Rh matrix was used for the measurement. In order to minimize saturation effects, the sample concentration, determined by atomic absorption, was kept under 2 mM.

The Mössbauer resonant absorption (A) was calculated from the area enclosed between the normalized Mössbauer spectrum and the nonresonant background. This method is particularly useful because the area is independent of the source line shape and instrumental vibrations and less sensitive than the depth of the adsorption to saturation effects (Lang, 1963; Morup & Both, 1975). The resonant absorption was corrected for thickness effects by using the method described by Lang (1963). The center shift (CS) was determined by the first moment of the Mössbauer spectrum and reported relative to metallic iron at 298 K. In order to obtain the required precision, the duration of each measurement was designed to accumulate a spectrum with $S/N > 30$. The quality of the spectra is illustrated in Figure 1 which displays the Mössbauer spectrum of an HbO_2 sample at 135 K. The reported error for the center shift was found to be consistent with a second set of measurements performed 6 months later on fresh samples.

Results

Figures 2 and 3 display the temperature profile of the center shift for Hb and HbCO. The CS values for HbO_2 were within the indicated error of the HbCO results at each temperature. In all cases, CS decreases at higher temperature and displays the expected linear behavior in the high-temperature regime. A comparison of the CS for the three complexes reveals a 4-fold higher value for Hb (0.930–0.860 mm s^{-1}) than for HbO_2 and HbCO (0.258–0.212 mm s^{-1}). This difference has been attributed to the change in the iron spin state from $S = 2$ for Hb to $S = 0$ for HbO_2 and HbCO (Lang & Marshall, 1966). In the temperature regime where a linear dependence of CS with temperature is observed, Hb displays a greater temperature dependence. Polynomial curve fitting of the data imparts a linear coefficient of $-4.2\ (3) \times 10^{-5}$ cm s^{-1} K^{-1} for HbO_2 and HbCO and $-6.5\ (4) \times 10^{-5}$ cm s^{-1} K^{-1} for Hb. The data for Hb are consistent with the reported CS temperature dependence of deoxymyoglobin (Mb) (Reinisch et al., 1985). Interestingly, the temperature dependence for HbO_2 and HbCO is less than that of Hb, with similar CS values for both

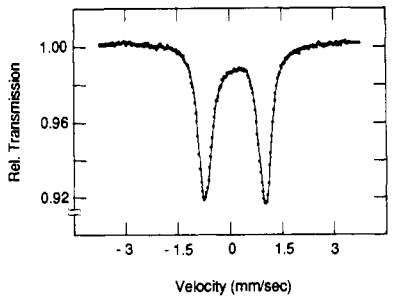

FIGURE 1: Experimental Mössbauer absorption spectrum of ^{57}Fe-enriched HbO_2 at 135 K. (The solid line represents the theoretical fit.)

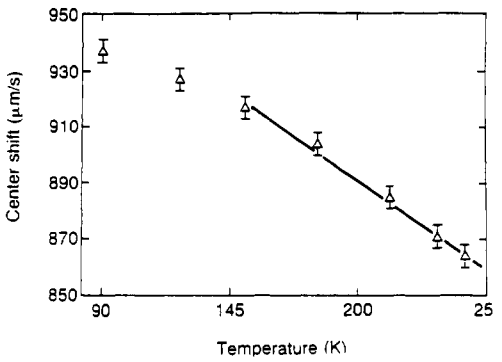

FIGURE 2: Center shift of [^{57}Fe]deoxyhemoglobin vs temperature. The solid line represents the linear regression in the 150–250 K temperature range.

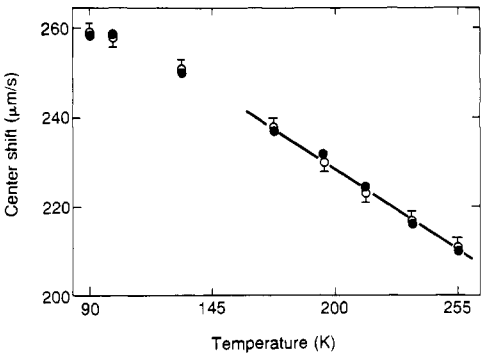

FIGURE 3: Center shift of [^{57}Fe](carbonmonoxy)hemoglobin (○) and oxyhemoglobin (●) vs temperature. The solid line represents the linear regression in the 150–250 K temperature range.

liganded hemoglobins over the entire temperature range.

Figures 4 and 5 show the temperature dependence of ln A for all three hemoglobin complexes. A decrease in the resonant absorption (A) is observed as a result of phonon excitations with increasing temperature. A deviation from linearity is observed above T_{cr} due to the excitation of conformational fluctuations. However, a diversity in the extent of the temperature dependence among the various complexes is easily noticed. In the "solid state regime" below T_{cr} within the temperature range 130–175 K, HbO_2, Hb, and HbCO exhibit linear regression of –0.0033 (3), –0.0037 (3), and –0.0064 (7) K^{-1}, respectively. The absolute value of these coefficients is proportional to $\langle x^2 \rangle_T$ and therefore portrays the flexibility experienced at the iron site. Surprisingly, HbCO, which possesses the same liganded relaxed "R" conformation as HbO_2, exhibits a softer lattice at the active site. It should, however, be noted that $\langle x^2 \rangle_T$ represents only displacements which are measurable by the lifetime of the excited nuclear level, namely, restricted to displacements which occur within a characteristic time $\tau \leq 100$ ns.

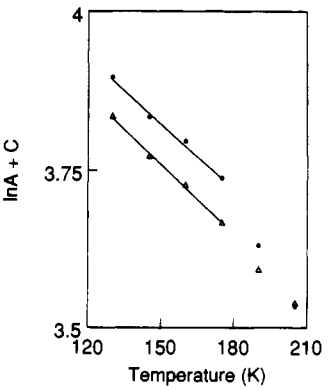

FIGURE 4: ln A of [^{57}Fe]deoxyhemoglobin (▲) and [^{57}Fe]oxyhemoglobin (●) vs temperature. The solid line represents the linear fit in the 130–180 K temperature range.

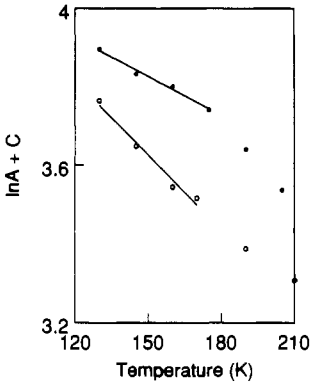

FIGURE 5: ln A of [^{57}Fe]oxyhemoglobin (●) and [^{57}Fe](carbonmonoxy)hemoglobin (○) vs temperature. The solid line represents the linear fit in the 130–180 K temperature range.

Excitation of conformational fluctuations which induce slow motion (with 1 ns $< \tau <$ 100 ns) results in the emergence of "broad" absorption lines in the Mössbauer spectrum (Parak & Reinisch, 1986). This "broad" component has been shown to increase in intensity as the temperature is raised at the expense of the ordinary "narrow" component. Thus, the temperature dependence of the intensity of the "narrow" component probes slow and fast movements.

Discussion

The Center Shift. The center shift consists of two contributions (Gütlich et al., 1978):

$$CS = IS + SODS \quad (1)$$

The isomer shift (IS) is a linear function of the electron density at the nucleus [$\psi^2(0)$] and therefore sensitive to the oxidation state, the electron configuration, and the nature of the bonds of the resonant atom to its ligands. A temperature-dependent isomer shift implies a concomitant temperature-dependent alteration of the electronic state.

The second contribution is the second-order Doppler shift (SODS) which originates from the relativistic shift in energy experienced by the nucleus due to its movement. This contribution is given by

$$SODS = -E_\gamma \langle v^2 \rangle_T / 2c^2 \quad (2)$$

where $\langle v^2 \rangle_T$ is the mean square velocity of the resonant atom and E_γ is the energy of the γ-radiation. According to the equipartition theorem, in the high-temperature range, one can write for the mean square velocity:

$$\langle v^2 \rangle_T = 3k_B T / M \quad (3)$$

where k_B is the Boltzmann constant and M the mass of the

resonant atom. By comparing eq 2 and 3:

$$\mathrm{SODS} = -3E_\gamma k_B T/2c^2M \quad (4)$$

These two contributions are addressed in the course of the discussion.

(*A*) *Effective Mass.* Deoxyhemoglobin (Hb) exhibits a CS temperature dependence of -6.5×10^{-5} cm s^{-1} K^{-1} (Figure 2), only slightly smaller than the SODS value of -7.1×10^{-5} cm s^{-1} K^{-1} expected for ^{57}Fe. Hb is, however, known to contain low-lying electronic states (Trautwein et al., 1975; Eicher et al., 1976; Bade & Parak, 1976; Bacci, 1978) which can contribute a temperature-dependent isomer shift to the observed CS. Apparently, these excited electronic levels, which mainly involve switching among the 3d orbitals, exhibit a small effect on electron density at the iron. In HbCO, where no low-lying electronic states exist (Case et al., 1981), one would expect a CS temperature dependence similar to that of the SODS. Surprisingly, the results indicate a distinctly different slope of -4.1×10^{-5} cm^{-1} s^{-1} K^{-1} (Figure 2).

For many simple solids, in which the intermolecular bonding forces are appreciably weaker than the intramolecular forces, M in eq 4 is replaced by an effective mass (M_{eff}) equal to the molecular weight of the monomeric units making up the solids (Herber, 1985). The smaller slope obtained for HbCO can therefore be attributed to an altered effective mass. Substituting the experimental slope of Figure 3, one can calculate through eq 4 an effective mass (M_{eff}) of 99 g/mol for the iron in HbCO. This value is consistent with an effective mass which includes at least the iron and CO. Support for the relevance of this model to Hb–Fe–CO bonds comes from resonance Raman experiments on HbCO which show stretching frequencies of 510 and 220 cm^{-1} (Spiro, 1983) for the Fe–CO and Fe–N (His) bonds, respectively, indicating much stronger bond with the CO than the histidine. The out of plane Fe porphyrin bonds are also thought to be weaker than the Fe–CO bond.

These results indicate that the exogenous CO molecule in HbCO—which is strongly coupled to the iron and forming no other bonds to the rest of the hemoglobin molecule—constitutes part of the resonant mass. Therefore, the Mössbauer center shift monitors modes of vibration in which the iron and its CO ligand are moving together as a single unit. Since the strength of the Fe–CO bond relative to the other Fe coordination groups is similar for CO and O_2, a parallel increase in the effective mass is expected. Although both HbCO and HbO_2 are considered low-spin Fe^{2+} complexes, there have been experimental and theoretical indications of the presence of low-lying electronic levels in HbO_2. The CS similarity of both complexes over the entire temperature range suggests that these excited levels have small contributions to the electron density at the nucleus.

(*B*) *Oxidation State of the Iron.* The nature of the oxygen bond to the iron in the heme protein has been the subject of intensive study by a large number of investigators (Rifkind, 1973). Different modes of binding were proposed in which the iron was assigned an oxidation state of 2+, 3+, and 4+. An interesting attempt to combine the ferrous Pauling model (Perutz et al., 1982) and the ferrous superoxide Weiss model (Cerdonio et al., 1985) was suggested by Tsai et al. (1981). Their model postulated a quantum mixture of ferrous and ferric states of a Mulliken's electron-donor acceptor type for the ground state of HbO_2. The temperature dependence of the Mössbauer center shift and quadrupole splitting observed for HbO_2 was explained in terms of modifications in the mixture of the oxidation states induced by the anharmonicity of the oxygen–iron binding potential well.

Such a change in the oxidation state of the iron for HbO_2 predicts a significant difference in the IS between HbO_2 and HbCO, in which the iron is known to be in the Fe^{2+} state. Our observations which indicate the same CS for both complexes over the entire temperature range are inconsistent with the model described above and suggest a similar oxidation state for the iron center of both complexes. This conclusion is supported by a magnetic field Mössbauer study at 4.5 T of oxymyoglobin (MbO_2) crystals (Maeda et al., 1981), which exhibits the same Mössbauer parameters as HbO_2. In this study, no variations in the high magnetic field spectra were detected in the temperature range between 4 and 130 K.

The Resonant Absorption (*A*). The resonant absorption obtained from the experimental spectrum is proportional to the recoil-free fraction f. For f, one can write

$$f = \exp(-\langle x^2\rangle_T/\lambda\!\!\!^{-2}) \quad (5)$$

where $\lambda\!\!\!^{-}$ is the reduced wavelength of the γ-radiation. Using the Thirring expansion to calculate the weighted average of the mean square displacement, one can write for the high-temperature range (Housley & Hess, 1966; Grow et al., 1978):

$$\langle x^2\rangle_T = (k_BT/M)[\mu(-2) + (1/12)(\hbar/k_BT)^2 - (1/720)(\hbar/k_BT)^4\mu(2)] \quad (6)$$

where $2\pi h$ is the Plank constant; $\mu(n)$ is the nth frequency moment of the lattice which is given by the expression

$$\mu(n) = \int G(\omega)\omega^n \, d\omega;\ \mu(0) = 1 \quad (7)$$

where $G(\omega)$ is the normalized phonon density of states function. In order to calculate $G(\omega)$, we have used the Einstein model which despite its simplicity has successfully explained the specific heat of most solids in the high-temperature range. In this model, the lattice is treated as an ensemble of independent harmonic oscillators of a single frequency ω_E given by

$$\omega_E = (F/M)^{0.5} \quad (8)$$

where F is the restoring force per unit of displacement and M is the mass. Accordingly

$$G(\omega) = \delta(\omega - \omega_E) \quad (9)$$

Combining eq 7–9, one can get for the Einstein frequency moment

$$\mu E(n) = (F/M)^{0.5n} \quad (10)$$

Taking the first approximation of the Thirring expansion and substituting in eq 5, one can write

$$\mu(-2) = (-\lambda\!\!\!^{-2}M/k_B)(\mathrm{d}\ln f/\mathrm{d}T) \quad (11)$$

In order to calculate $\mu(-2)$, the relative changes in f as a function of temperature are required, and, therefore, $\mathrm{d}\ln f/\mathrm{d}T$ can be substituted for by $\mathrm{d}\ln A/\mathrm{d}T$ where A is given in arbitrary units.

By substituting the value of μ_E in eq 11

$$F = -k_B\lambda\!\!\!^{-2}(\mathrm{d}\ln A/\mathrm{d}T)^{-1} \quad (12)$$

which for the 14.4-keV line of ^{57}Fe is

$$\sim -73.8(\mathrm{d}\ln A/\mathrm{d}T)^{-1}\ \mathrm{dyn/cm} \quad (13)$$

Taking the high-temperature harmonic approximation for the hemoglobin complexes in the "solid state regime", namely, between 130 and 175 K, and substituting the experimentally determined values of $\mathrm{d}\ln A/\mathrm{d}T$ in eq 13, one can show that the restoring force is 2.2 (2) $\times 10^4$, 2.0 (2) $\times 10^4$, and 1.2 (2) $\times 10^4$ dyn/cm for HbO_2, Hb, and HbCO, respectively.

It is important to note that the intensity analysis used to calculate the restoring force was restricted to the "narrow"

component alone. Although "broad" components, which originate from the excitation of "damped oscillations" (Parak & Knapp, 1984; Nowik et al., 1985), were experimentally undetected in the discussed temperature range, it is still possible that they account for the loss in intensity as temperature is raised. In such a case, the application of the Einstein model represents an effective harmonic force approximation for the compounded motion of the iron.

The structural differences in the coordination of iron for unliganded deoxyhemoglobin and liganded hemoglobin (Baldwin & Chothia, 1979) are expected to influence the observed restoring force. However, the appreciably greater restoring force for HbO_2 than HbCO is unexpected since the iron coordination geometries for both complexes are very similar (Heidner et al., 1976; Shaanan, 1983), particularly in terms of the binding to the porphyrin and proximal histidine. This difference must therefore be attributed to the strong coupling of the motion of the iron with that of the exogenous ligand. It appears that because of this coupling with the exogenous ligand the Mössbauer resonance is able to probe the potential energy surface in the ligand pocket. The oxygen bound in the "bent" orientation (Grow et al., 1978) is thought to fit into the favored configuration of the ligand pocket. HbO_2 therefore experiences a relatively steep potential well with a strong restoring force. On the other hand, the preferred linear configuration for CO binding to hemes results in steric interactions with the ligand pocket (Heidner et al., 1976; Moffat et al., 1979). These interactions influence both the Fe–CO bond angle (Herber, 1985) and also the globin configuration (Moffat et al., 1979). The interplay between the ligand orientation and the globin configuration results in a broader, shallower potential energy well which translates into a weaker restoring force observed in our study. This restoring force detected by the ligand protruding into the heme pocket complements recent infrared (IR) results on (carbonmonoxy)-hemoglobin and myoglobin. These IR studies indicate multiple states for both CO bound to the iron (Alben et al., 1982) and photolyzed CO where the CO is still in the ligand pocket (Alben et al., 1982; Bianconi et al., 1985). These multiple states are consistent with a broad potential energy well where interconversion between related substates is indicative of a weak restoring force.

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