Low-Temperature Formation of a Distal Histidine Complex in Hemoglobin: A Probe for Heme Pocket Flexibility

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ABSTRACT: Pocket dynamics of horse deoxyhemoglobin and methemoglobin in the temperature range from 80 to 260 K is investigated. In both hemoglobins reversible conversion to a low-spin iron complex is observed at temperatures as low as 210 K. Electron spin resonance (ESR) and Mössbauer data assigned this low-spin iron complex to the coordination of N^{τ}-His-E_7 as a sixth nitrogenous ligand. The bonding of this ligand located 4 Å from the iron indicates the presence of a thermally available conformation that exhibits a high degree of flexibility in the heme pocket. In deoxyhemoglobin, the formation of the bis(histidine) complex was accompanied by excitations of conformational fluctuations manifested through the temperature dependence of the Mössbauer–Lamb factor. The rate for the formation of this complex, with an associated energy barrier (>60 KJ mol^{-1}), is shown to serve as an index of heme pocket flexibility. Measurements performed on partially liganded (carbonmonoxy)hemoglobin indicate that partial ligation enhances conversion of the unliganded subunits to the bis(histidine) complex, suggesting that pocket dynamics is affected by subunit interactions.

It has become increasingly evident in recent years that proteins are not rigid systems and conformational fluctuations frequently play a major role in their biological activity (Karplus & McCanmon, 1981; Westhof et al., 1984; Tainer et al., 1984). The study of these dynamic processes at cryogenic temperatures offers experimental advantages in terms of slowing down fluctuations, gradual excitations of different fluctuation modes, enhancing the population of some conformational intermediates, and allowing the application of more powerful experimental techniques. Recent comparative X-ray (Frauenfelder et al., 1979), visible (Doster et al., 1982), and Raman (Ondrias et al., 1983) measurements seem to establish the relationship of the cryogenic conformational fluctuations to functional hemoglobin at physiological temperatures. Mössbauer spectroscopy, which is applicable to both Fe(II) and Fe(III) hemoglobins (Lang & Marshall, 1966), is a particularly powerful method in studying dynamics of the heme iron (Keller & Debrunner, 1980; Parak et al., 1981).

The X-ray structures for hemoglobin indicate that the iron is bonded to a histidine on the proximal side of the heme and located more than 4 Å away from another histidine in the ligand pocket (Perutz et al., 1968; Bolton & Perutz, 1970; Fermi et al., 1984). These structures further show that appreciable flexibility at the entrance to the ligand pocket is required to permit access of ligands to the binding site (Case & Karplus, 1979). There have been a number of reports in the literature for complexes involving the distal histidine and the iron (Peisach et al., 1973; Mayo et al., 1983; Papaefthymiou et al., 1975; Rein et al., 1971). However, these complexes which require an appreciable shortening of the iron–distal histidine distance have been attributed to denatured nonfunctional forms of hemoglobin.

In this paper we demonstrate that the bis(histidine) complex is a thermally available reversible low-entropy conformation of functional hemoglobin. Even though this complex may be nonfunctional, the extent of this complex in the temperature range 210–250 K is shown to provide a valuable index for the functionally crucial dynamics on the distal side of the heme.

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). The ^{57}Fe deoxyhemoglobin was prepared from the methemoglobin in a 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. In this way potential damage provided by the reaction products of oxygen and dithionite was minimized.

A comparison of the ^{57}Fe-reconstituted and native methemoglobin was made by isoelectric focusing as well as visible and electron spin resonance spectroscopy. The reversible oxygenation of the reduced ^{57}Fe deoxyhemoglobin was checked by visible and Mössbauer spectroscopies.

The samples for Mössbauer measurements were transferred to a Lucite holder that provides a disk-shape space for the solution 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 source of 100 mCi of ^{57}Co diffused into Rh matrix was used for the measurement. The theoretical fit of the data which is shown as a solid line in the figure was obtained by assuming Lorentzian shape for the absorption lines. The isomer shift values are given relative to metallic iron.

Results

Deoxyhemoglobin. The 14 keV γ transmission Mössbauer spectra of ^{57}Fe-enriched horse deoxyhemoglobin at various temperatures are shown in Figure 1. Figure 1a displays an 87 K Mössbauer spectrum of a sample that was quickly frozen prior to the measurement by immersing it into liquid nitrogen. The spectrum exhibits a prominent quadrupole pattern with an isomer shift of 0.93 mm/s and quadrupole splitting of 2.34 mm/s. These parameters are consistent with those reported

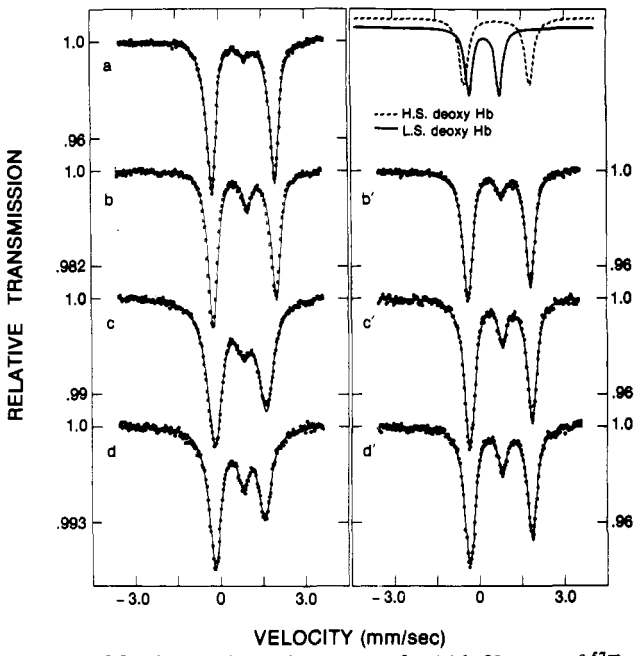

FIGURE 1: Mössbauer absorption spectra for 14 keV γ ray of ^{57}Fe-enriched horse deoxyhemoglobin (a) at 87, (b) at 210, (c) at 240, and (d) at 260 K. Spectra b′, c′, and d′ obtained at 87 K after being incubated for 14 h at 210, 240, and 260 K, respectively. Circles represent experimental data, and the solid line represents the theoretical fit.

Table I: Mössbauer Parameters for Horse Hemoglobin

	temp (K)	isomer shift (mm/s)	quadrupole splitting (mm/s)
H.S. deoxyHb[a]	240	0.85 (2)	1.89 (2)
L.S. deoxyHb[b]	240	0.45 (2)	1.08 (2)
HbCO	240	0.22 (2)	0.42 (2)
metHb component A	195	0.38 (1)	1.43 (2)
metHb component B	195	0.26 (1)	1.65 (2)
metHb component C	195	0.22 (1)	2.12 (2)
pyridine hemochrome	240	0.40 (2)	1.18 (2)

[a] H.S., high spin. [b] L.S., low spin.

by other investigators for human and rat deoxyhemoglobin (Papaefthymiou et al., 1975; Lang et al., 1966). As the temperature is increased (Figure 1b–d) one can seen the emergence of a new absorption peak between the previous two peaks of deoxyhemoglobin accompanied by increased absorption in the region of the negative velocity peak. This observation indicates that deoxyhemoglobin consists of two distinct species of iron, each of them represented by a doublet in the Mössbauer spectrum (Figure 1). Analysis of the data reveals that the new Fe(II) species has an isomer shift and quadrupole splitting consistent with that of a low-spin Fe(II) complex. The values for the parameters of both deoxy species at 240 K are given in Table I.

The formation of a low-spin complex implies the coordination of a sixth ligand. This ligand can be identified as the N^{τ}-His-E_7, which is the only available globin-coordinating group on the distal side of the heme iron. Species with the same Mössbauer parameters as those found for this complex have previously been assigned by other workers to a complex involving both the proximal histidine and distal histidine (Papaefthymiou et al., 1975; Eicher et al., 1976; Mayo et al., 1983). This conclusion is confirmed by the similarity of the Mössbauer parameters with that of a pyridine hemochrome where the porphyrin Fe is coordinated to two axial nitrogenous ligands (Table I).

The Mössbauer intensity of a given component a, determined

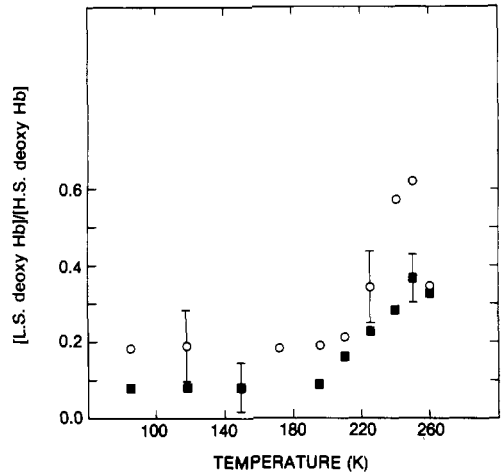

FIGURE 2: Relative concentration of the low-spin horse deoxyhemoglobin complex vs. temperature of 100% deoxyHb (□) and 25% partially liganded HbCO (○).

by the area A_a of the corresponding absorption in the spectrum, is given by

$$A_a(T) = B C_a(T) f_a(T) \quad (1)$$

where B is a constant independent of the compound and temperature. $C_a(T)$ and $f_a(T)$ are the concentration and the Lamb–Mössbauer factor, respectively, of component a at temperature T. Factor f decreases with temperature and depends on the bond strength of the resonant atomic nucleus to its matrix. For $f_a(T)$ one can write

$$f_a(T) \propto \exp[-(4\pi^2\langle X^2\rangle_T/\lambda^2)] \quad (2)$$

where λ is the wavelength of the γ ray for the 14 keV γ radiation of ^{57}Fe, $\lambda = 0.86$ Å). The temperature dependence of f is introduced through the mean square displacement $\langle X^2\rangle$ of the resonant nucleus.

Expression 1 shows that the temperature dependence of the relative intensity of a given compound is not exclusively determined by changes in the concentration. Therefore, in order to determine the change in concentration with respect to temperature, it was necessary to obtain additional spectra of samples recooled to $T' = 87$ K after being incubated at the appropriate temperatures (Figure 1b′–d′).

Assuming that no conversion between the two deoxy components occurs in the process of rapid cooling from T to T' = 87 K and that f_a does not depend on C_a, one can write for the intensity of component a in the primed spectra

$$A'(T) = B C_a(T) f_a(T') \quad (3)$$

where $C_a(T)$ is the concentration of component a of a sample that had been incubated at temperature T.

Figure 2 displays the relative concentration of the Fe(II)–bis(histidine) complex vs. temperature as calculated from eq 3. At 210 K the concentration begins to increase and reaches its maximum concentration around 250 K. The reversibility of the formation of this species was demonstrated by the observation that thawing the sample from any temperature, and again rapidly cooling it back to 87 K, produces a spectrum identical with that of Figure 1a. Figure 1a and the plateau in Figure 2 indicate the presence of a small amount of the bis(histidine) complex for samples rapidly cooled from room temperature to below 200 K. This signal is not detected when these samples are completely reacted with oxygen or carbon monoxide and, thus, cannot be attributed to denatured hemoglobin. Therefore, the inability to obtain a spectrum of deoxyhemoglobin without any low-spin component can be

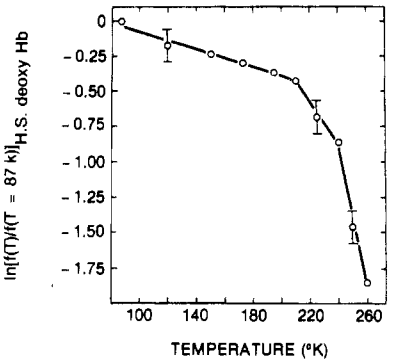

FIGURE 3: Normalized plot of $\ln f(T)$ vs. temperature for the high-spin horse deoxyhemoglobin complex.

explained either by the rapid formation of the bis(histidine) complex during the cooling process or by the presence of a low concentration of this complex even at room temperature.

For proteins one can write (Frauenfelder et al., 1979)

$$\langle X^2\rangle_T = \langle X^2\rangle_T^v + \langle X^2\rangle_T^c + \langle X^2\rangle_T^d \qquad (4)$$

where $\langle X^2\rangle_T^v$, $\langle X^2\rangle_T^c$, and $\langle X^2\rangle_T^d$ are the contributions to the mean square displacement from lattice vibrations, conformational fluctuations, and diffusive motions, respectively.

At low temperature phonons dominate $\langle X^2\rangle$, and the protein behaves as a solid lattice. Above a critical temperature where a continuum of vibrational states can be considered, a plot of $\ln f$ vs. T will exhibit linear behavior. As T increases the contribution of conformational fluctuations, $\langle X^2\rangle_T^c$ becomes noticeable, and a break from linearity in the $\ln f(T)$ profile is observed. Diffusive motion at still higher temperatures would produce an additional "break" in the slope.

Figure 3 displays the normalized $\ln f$ vs. T for high-spin deoxyhemoglobin. The values of f, at each temperature, were calculated by taking into account the temperature dependence of the concentration (eq 1), due to formation of the bis(histidine) complex. A change in a slope around 210 K is noticed. Since diffusive motion is not observed for hemoglobin until above 250 K (Mayo et al., 1983), this break is attributed exclusively to conformational fluctuations.

No formation of a bis(histidine) complex is observed for liganded Fe(II) hemoglobins such as HbO_2 or HbCO. With Fe(III) hemoglobin no additional hemoglobin complexes were found for strong ligands such as CN^- or azide. However, the formation of an additional species was observed with weak Fe(III) ligands such as H_2O and OH^- (Levy et al., 1982).

Methemoglobin. The slow relaxation of the paramagnetic Fe(III) in methemoglobin [relative to the high-spin Fe(II)] causes a broad unresolved Mössbauer spectrum at 85 K. Therefore, we have carried out the measurements at 195 K, in the fast relaxation range, where a better resolution is achieved and the intensity of each component can be more accurately determined. In a previous publication (Levy et al., 1982) we have shown that the 195 K methemoglobin Mössbauer spectra at various pHs consist of three components: a relaxed high-spin component (A), displayed by an asymmetric doublet, and two low-spin components (B, C), displayed by two symmetric doublets (Table I and Figure 4). Component C was found to form at all pHs between 6.0 and 8.6. Components A, B, and C have been assigned by comparative ESR measurements to the aquomethemoglobin complex, the low-spin hydroxide complex, and a low-spin Fe(III)–bis(histidine) complex, respectively. The rhombic and tetragonal distortion deduced from the g values of component C were found to be characteristic of a nitrogenous sixth ligand (Peisach et al., 1973). Similar parameters have been reported for hemichromes were distal histidine binds to the iron (Rachmilewitz et al., 1971; Peisach et al., 1975; Carrell et al., 1966). The effect of temperature and time of incubation on the shape of the pH 6.9 methemoglobin Mössbauer spectrum at 195 K is shown in Figure 4. In the initially rapidly cooled sample (Figure 4a), the shoulder in the negative velocity peak is due to component C. It can be seen that during incubation at 210 K (Figure 4b,c) there is an increase in the relative intensity of component C. In the sample incubated for 7 days (Figure 4d), component C becomes the dominant complex. From the time dependence of the relative concentration of the Fe(III)–bis(histidine) complex (component C) calculated from the fit of the spectra, one can measure the rate of the reaction

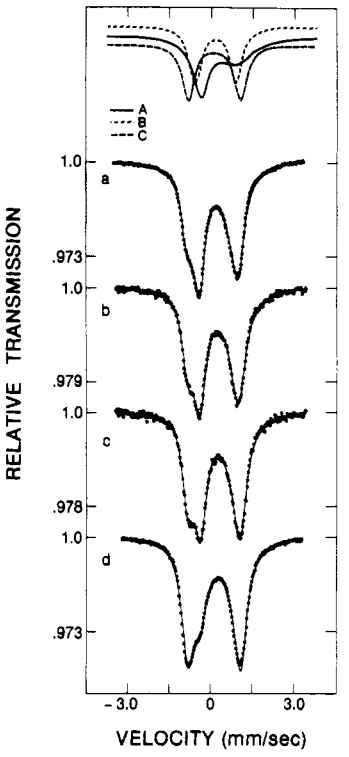

FIGURE 4: Mössbauer absorption spectra for 14 keV γ ray of pH 6.9 horse methemoglobin performed at 195 K. (a) Immediately after being cooled from room temperature; (b) same as (a) after incubation at 210 K for 1.5 h; (c) same as (b) after incubation at 210 K for 14 h; (d) room temperature sample incubated at 215 K for 7 days.

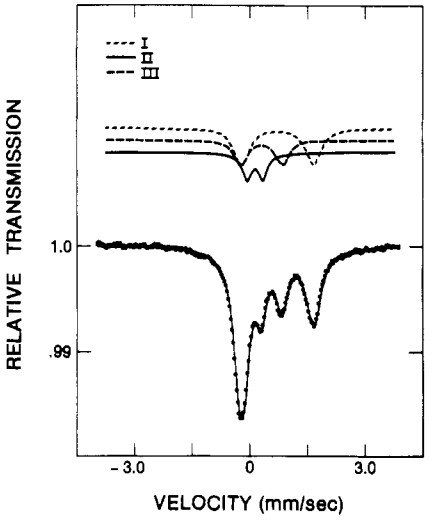

FIGURE 5: Mössbauer absorption spectrum for 14 keV γ ray of partially liganded HbCO at 240 K (see text).

as well as its temperature dependence.

Partially Liganded Hemoglobin. Figure 5 displays the Mössbauer spectrum of a partially liganded (carbonmonoxy)hemoglobin (HbCO) at 240 K. As shown at the upper part of the figure, the absorption feature consists of three doublets. Doublet I is the usual high-spin deoxyhemoglobin (see Table I). Doublet II has an isomer shift of 0.22 mm/s and a small quadrupole splitting of 0.42 mm/s consistent with reported data for HbCO. Doublet III is identical with the Fe(II)–bis(histidine) complex (Table I) formed when the high-spin deoxyhemoglobin is incubated at temperature of 210 K and above (Figure 2). For the partially liganded sample, as shown for pure deoxyhemoglobin, this low-spin component is also formed after incubation at elevated temperatures.

As done for deoxyhemoglobin, Mössbauer spectra of the partially liganded hemoglobin were obtained at 87 K subsequent to each measurement at an elevated temperature. In this way it was possible to show that regardless of the incubation temperature the proportional absorption of the CO complex remained 25%. The bis(histidine) complex is thus formed exclusively at the expense of the high-spin deoxyhemoglobin. Correcting for the temperature dependence of the f factor and assuming that rapid cooling to 87 K does not change the concentration of the various species, a plot of the concentration of the bis(histidine) complex relative to the high-spin deoxyhemoglobin is compared with that found for pure deoxyhemoglobin in Figure 2. One can notice that in the partially liganded HbCO the bis(histidine) complex is formed to a greater extent and peaks at lower temperatures as compared to pure deoxyHb.

Discussion

The results on both deoxyhemoglobin and methemoglobin show the reversible formation of a low-spin complex involving the coordination of the N^{τ}-His-E_7.

Similar complexes have been previously reported under denaturing conditions produced by low pH (Peisach et al., 1973), due to the addition of various denaturants (Rachmilewitz et al., 1971; Peisach et al., 1975) or dehydration of the hemoglobin (Papaefthymiou et al., 1975). Such complexes have also been found with unstable hemoglobins such as hemoglobin Koln (valine β98 → methionine) (Carrell et al., 1966). The complexes formed under these conditions are for the most part not in dynamic equilibrium with the usual hemoglobin states.

Conversely, the appearance of this complex has been attributed to a nonreversible denatured hemoglobin conformation that is not a part of the various conformational substates thermally available from functional hemoglobin (Mayo et al., 1983). Our results indicate unequivocally that it is possible to form such complexes in frozen hemoglobin solutions that are completely reversible by thawing the sample. This reversal is distinguished from other reported reversible hemichromes that require reduction of the iron.

The X-ray structure for hemoglobin in both the T and R states shows that the shortest distance between the distal histidine and the iron is greater than 4 Å (Baldwin & Chothia, 1979). This static structure would seem to preclude complex formation with the distal histidine. There must, however, be adequate movement within this region to permit the formation of a low-spin complex with considerably shorter Fe–N distances. The necessary rearrangement of the protein in order to form the bond suggests a large energy barrier for the formation of this complex.

The conversion to the distal histidine complex at 210 K (Figure 2) shows that the appropriate conformational substrate

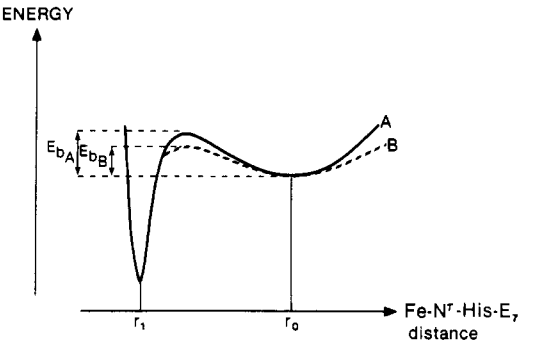

FIGURE 6: Potential energy diagram for the formation of the distal histidine complex.

which favors this complex is the lowest in energy. At lower temperatures the thermal energy is small compared to the required activation energy, and no appreciable complex formation is detected. An increase in conversion rate in the temperature range 210–250 K is associated with the increase in the thermal energy associated with increased dynamic freedom in the ligand pocket, which facilitates the formation of the distal histidine complex. The diminished levels of the bis(histidine) complex at 260 K and above indicate a higher multiplicity for the unbonded state.

A simple potential energy diagram for the formation of the bis-histidine complex is depicted in Figure 6. The narrow and deep well at the left represents the bis(histidine) complex. The minimum of this well corresponds to the iron–N^{τ} distal histidine bond length (7). The broad and shallow well A centered around the large iron–distal histidine distance (r_0) represents the high-spin deoxyhemoglobin or methemoglobin. The shape of this well is consistent with the preferred formation of this state at higher temperatures. The formation of the bis(histidine) complex involves the transition to the lower energy left well. The rate of the transition is determined by the barrier height, E_{bA}, and the temperature.

The barrier height (E_{bA}) for this reaction assuming an Arrhenius type reaction rate (k) is given by $E_b = R^2\, d \ln k/dT$. Although the long time required to obtain adequate statistics makes the Mössbauer technique particularly poor for measuring reaction rates, it was possible to obtain a lower limit for E_{bA} from our data. The best estimate is calculated from the low-temperature data where the slow reaction rates relative to the time of the measurement permit us to use a linear approximation. The results of the formation of the bis(histidine) complex in both deoxyhemoglobin and methemoglobin set a lower limit of 60 kJ mol^{-1} for E_b.

What is the origin of this energy barrier of 60 kJ mol^{-1}? Three major barrier factors known to contribute to this barrier are the change in the protein conformation, the flattening of the domed heme, and the movement of the iron to the porphyrin plane. Flash photolysis studies at low temperature provide estimates of these factors for the binding to iron of a free ligand already located in the heme pocket. These studies (Austin et al., 1975) report values ranging from 6 to 12 kJ mol^{-1} for different proteins. The unusually high barrier for the formation of the bis(histidine) complex cannot be attributed to changes in the required flattening of the heme or movement of the iron, which are expected to be similar for the formation of all low-spin complexes whether they be oxygen, carbon monoxide, or a histidine. The origin of this higher barrier for the indigenous histidine as opposed to that for exogenous ligands must therefore be attributed to a greater contribution of the protein that is associated with the movement and rearrangement of the distal side of the pocket.

The energy associated with this rearrangement determines the shape of the right well depicted in Figure 6. Enhanced flexibility of the ligand produces a shallower well (curve B, Figure 6) resulting in a lower energy barrier and thereby a higher formation rate of the histidine complex. Although the distal histidine complex may not be relevant to the functioning of hemoglobin, the rate of the complex formation nevertheless provides a valuable index for the flexibility of the ligand pocket.

The relationship of the pocket flexibility and the formation of the histidine complex is confirmed by comparing hemoglobin with myoglobin and leghemoglobin. Myoglobin with less dynamic freedom in the ligand pocket (Case, 1982) does not form a bis(histidine) complex (Levy et al., 1984) while the monomeric leghemoglobin which exhibits a high degree of pocket flexibility forms the bis(histidine) complex more readily (Appleby et al., 1982).

Furthermore, it is interesting to note that this flexibility appears to be associated with greater dynamics at other loci on the protein. Flash photolysis studies indicate that the ligands can move into and out of the pocket at 210 K where the appreciable flexibility was observed in our experiment. At the same temperature, Mössbauer measurements (see Figure 3) indicate the onset of the conformational fluctuation coupled to the movement of the iron. Each type of measurement detects a different range of motion. The decrease in the Lamb–Mössbauer effect corresponds to movement of the iron 0.1–0.2 Å, while the formation of the distal histidine complex corresponds to movement of 1–2 Å, and the entry of ligands into and out of the pocket probably involves even larger molecular movements.

Is there a possible relationship between protein flexibility and the ligand binding reaction? It has been shown in several recent papers (Tainer et al., 1984; Westhof et al., 1984) that at least for immunoglobins the flexible region of the molecule plays a functional role. It has also been argued (Cooper, 1984) that protein fluctuations could play a major role in enzymatic reactions and even in transmitting allosteric interaction.

The results from the partially liganded HbCO indicate an increase in the formation of the bis(histidine) complex relative to that of 100% deoxyhemoglobin. The detection of an appreciable effect at 25% CO where a significant fraction of hemoglobin is in the fully unliganded and fully liganded form suggests an appreciable reduction in the energy barrier, i.e., enhanced dynamics, for the partially liganded hemoglobin molecules (Figure 6).

These results suggest that protein dynamics can be induced in the unliganded pocket through subunit interactions. These dynamic factors may contribute to the cooperative interactions in addition to the static conformational changes emphasized in a number of models for heme–heme interaction.

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