

# Mössbauer Spectroscopy on Nonequilibrium States of Myoglobin: A Study of $\tau$ -t Relaxation

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**ABSTRACT** A frozen solution of <sup>57</sup>Fe-enriched metmyoglobin was irradiated by x rays at 77 K. Mössbauer spectra showed a reduction of Fe(III) high spin by thermalized electrons and a production of a metastable Fe(II) low spin myoglobin complex with H<sub>2</sub>O at its sixth coordination site. The relaxation of the intermediate was investigated by Mössbauer spectroscopy as a function of temperature and time. The relaxation process starts above 140 K and is fully completed at ~200 K. At temperatures between 140 and 200 K, the relaxation lasts for hours and is nonexponential in time. Up to 180 K, the process can be described satisfactorily by a gamma distribution of activation enthalpies with an Arrhenius relation for the rate coefficient. The temperature and time dependence of the Mössbauer parameters indicates structural changes in the active center of the protein as early as 109 K that continue for several hours at higher temperatures. Above 180 K, structural rearrangements involving the whole protein molecule lead to a shift and narrowing of the barrier height distribution.

## INTRODUCTION

The function of proteins depends essentially on their structure and the structural dynamics. Proteins acting as enzymes normally have two conformations. The binding of a substrate gives rise to a structural change. Also, the interaction of a transport protein like myoglobin with a ligand is accompanied by structural changes. They can be understood through two main steps. First, the active center reacts with the ligand and the heme iron turns into a new state. The entire protein globule is not able to alter its conformation very quickly. Therefore, an intermediate state may arise where the initial conformation of the globule coexists with a new state of the heme iron. During the second step, the whole protein molecule adapts its structure to the converted active center and finally reaches the new equilibrium conformation. The whole structural relaxation can be described by the picture of protein quakes (Ansari et al., 1985). A detailed study of the relaxation process can provide insight into the working mechanisms of proteins.

In the case of heme proteins, there are several ways to investigate nonequilibrium states, which can be generated at low temperatures. One possibility is flash photolysis of the ligated form of the protein. This technique was developed mainly by H. Frauenfelder and co-workers (Ansari et al., 1985, 1987; Austin et al., 1975; Doster et al., 1982; Steinbach et al., 1991; Nienhaus et al., 1992). The optical experiments on carboxy- and oxymyoglobin (MbCO and MbO<sub>2</sub>) have shown nonexponential kinetics of the ligand rebinding to the heme iron after photodissociation at low temperatures. This fact has led to the concept that an ensemble of molecules in

one conformation shows structural inhomogeneities and that each molecule is in its own conformational substate. Supplementary data to the rebinding kinetics have been obtained by Mössbauer studies of MbCO photodissociation (Winkler et al., 1990). The x-ray structure analysis at several temperatures (Frauenfelder et al., 1979, 1988; Parak et al. 1987) as well as hole-burning experiments (Köhler et al., 1988; Zollfrank et al., 1992) and EPR data (Bizzari and Cannistraro, 1993) strongly support the existence of conformational substates.

Another way to generate intermediates of heme proteins at low temperatures is the irradiation of frozen solutions by gamma- or x-rays (Blumenfeld, 1981; Prusakov et al., 1981, 1985, 1990; Parak and Prusakov, 1994). The radiolysis of the solvent produces thermalized electrons that can react with the heme even at 77 K because of their great mobility. Starting with metmyoglobin (metMb), the heme iron Fe(III) is reduced, but the "frozen" globin structure remains in its initial conformation. Thus, the protein is in a kinetically stabilized nonequilibrium state. A mixture of water with a polyatomic alcohol usually is used as a solvent in these experiments. The alcohol is an effective scavenger of hydroxyl radicals (Sonntag, 1987). These are also produced at the radiolysis and can compete efficiently with the heme in the reaction with the thermalized electrons.

Mössbauer spectroscopy is a valuable tool to investigate structural fluctuations of proteins (Parak and Formanek 1971; Parak et al., 1981, 1982; Bauminger et al., 1983; Parak and Frauenfelder, 1993). This method has also been used to study the nonequilibrium states generated at low temperatures by gamma-irradiation of the met-aquo forms of mouse hemoglobin (Prusakov et al., 1981), insect hemoglobin (erythrocrucorin) (Prusakov et al., 1985), and sperm whale myoglobin (Prusakov et al., 1990; Parak and Prusakov, 1994). It allows the determination of the iron state in the metmyoglobin molecule before irradiation, in the intermediate and in the final equilibrium conformation. It was shown

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that the metastable conformation produced after low temperature irradiation contains Fe(II) low spin. With increasing temperature, the molecules relax into the equilibrium state of deoxygenated myoglobin (deoxyMb) with Fe(II) high spin. This relaxation is an example of a *r-t* transition. The study of this process allows us to determine the energy barriers that the protein molecule has to overcome during the relaxation and provides deeper insight into conformational changes of proteins. In this paper, we investigate the temperature and the time dependence of the relaxation.

## MATERIALS AND METHODS

Sperm-whale metmyoglobin was chemically enriched with the  $^{57}\text{Fe}$  isotope according to Parak et al. (1987) and crystallized. The samples were obtained by dissolving the metMb crystals in a water/glycerol mixture (1:1, v/v) at pH 6.6 adjusted by 0.05 M potassium phosphate buffer. The protein concentration was  $\sim 80$  mg/ml. The samples with a volume of 1.1 ml and a thickness of 4 mm were tightly closed in PVC holders by indium-sealed mylar windows. The metMb samples were irradiated at 77 K by x rays from a conventional x-ray tube with a Cu or Mo anode. The irradiation was carried out from both sides of the samples to provide homogeneous x-ray absorption through the whole sample depth. The total illumination lasted for 90 h.

After the sample irradiation, two types of Mössbauer experiments were carried out to investigate the relaxation process. 1) The temperature dependence of the structural relaxation was determined using the following temperature cycling procedure: the sample was heated in the Mössbauer cryostat to a temperature  $T_i > 80$  K with a rate of 2–3 K/min and kept at  $T_i$  for 45 min. Then the sample was cooled with a rate of 2–5 K/min, and its Mössbauer spectrum was measured at 80 K. The cycle was repeated with  $T_{i+1} > T_i$ . 2) To measure the kinetics of the relaxation, the sample was heated several times to the same temperature,  $T_i$ , and was kept at this temperature for different time intervals. The Mössbauer spectra again were measured at 80 K to exclude relaxation processes during the measurement and to allow a comparison of the areas of the different iron species in the spectra after the thermal cycles.

A  $^{57}\text{CoRh}$  source and a conventional electromagnetic drive system with a sinusoidal velocity profile were used to measure the Mössbauer spectra. A bath cryostat provided the required temperature of the samples with an error better than  $\pm 2$  K.

For the computation of the Mössbauer spectra, the transmission integral was solved numerically. The often used fit with the approximation of a sum of Lorentzians is adequate only for "thin" absorbers with homogeneous states of the iron atoms. This condition is not fulfilled in our case. The samples are fairly "thick" and contain several iron species with overlapping spectral lines. By comparison, we showed that the transmission integral yields great advantages for the correct description of the spectra. Besides the values of the absorption area, the quadrupole splitting (QS) and the isomer shift (IS) of each spectral component the transmission integral fit procedure allow the determination of the Gaussian broadening of the Mössbauer lines, which indicates an inhomogeneous distribution of the relevant iron state. The Lamb-Mössbauer factors of the different iron species were assumed to be equal at 80 K. The values of the isomer shifts are given relative to metallic iron ( $\delta\text{-Fe}$ ) at room temperature.

## RESULTS AND DISCUSSION

Fig. 1 gives the Mössbauer spectra of the metMb sample before and immediately after the low temperature x-ray irradiation. The nonirradiated sample shows the paramagnetic relaxation spectrum that is typical for a Fe(III) high spin heme (Fig. 1 *a*). The irradiation yields a dramatic change of the Mössbauer spectrum (Fig. 1 *b*). It can be fitted by three quadrupole doublets. The main component (89% of the

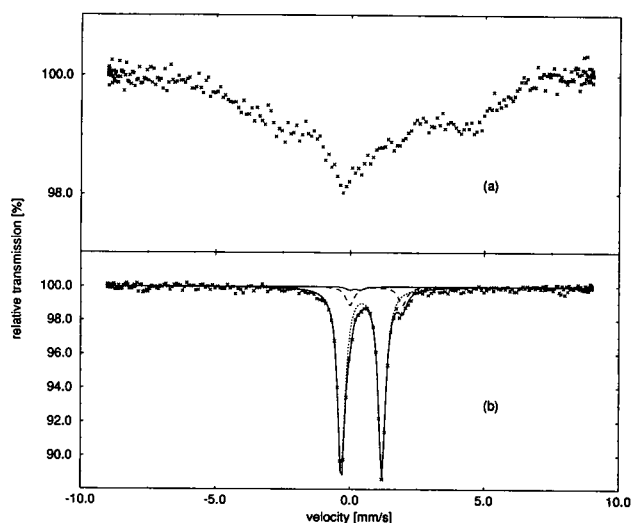


FIGURE 1 Mössbauer spectrum at  $T = 80$  K of metMb before (*a*) and after (*b*) irradiation by x rays at 80 K. The main doublet in *b* represents the heme Fe(II) low spin complex. Solvent: 50% glycerol/buffer, pH 5.6.

whole area) shows the hyperfine parameters of a six-coordinated heme Fe(II) low spin (compare Table 1). The spectrum contains two additional doublets. The doublet explaining 8% of the total area has parameters close to those of deoxyMb. The Mössbauer parameters of the least intense doublet (3% of the total area) are typical for MbCO.

In metmyoglobin, the high spin heme Fe(III) is sixfold coordinated. Four coordinates are occupied by the nitrogen atoms of protoporphyrin IX. One axial ligand is the imidazole nitrogen of the proximal histidine F8. At the distal site of the heme, a water molecule occupies the second axial coordination (Fig. 2). The Mössbauer data show that after x-ray irradiation the initial Fe(III) is reduced by the thermalized electrons. In most of the molecules, the Fe(II) low spin form is obtained, indicating that the initial six coordinations, including the one with the water molecule, are retained. The Fe(II)-H<sub>2</sub>O coordination is very unusual for heme proteins. It is stabilized by steric restraints of the frozen conformation in metmyoglobin because the relaxation into the equilibrium conformation is strongly hindered at 77 K. Nevertheless, a minority of  $\sim 10\%$  of the molecules relaxes into the deoxy conformation even at 77 K as seen from the Mössbauer spectra. This, again, is an indication for the heterogeneity of an ensemble of molecules in one conformation. Some of the metMb molecules have a very low energy barrier so that they relax immediately into the equilibrium conformation after reduction. CO molecules produced by the ir-

TABLE 1 Mössbauer parameters at  $T = 80$  K of iron species produced after x-ray irradiation of the MetMb sample at 80 K

species Nr	IS <sub><math>\alpha\text{-Fe}</math></sub> (mm/s)	QS (mm/s)	Iron state
1	$0.66 \pm 0.03$	$1.53 \pm 0.02$	Fe(II) low spin
2	$1.08 \pm 0.03$	$2.04 \pm 0.02$	Fe(II) high spin
3	$0.29 \pm 0.02$	$0.39 \pm 0.02$	MbCO
deoxyMb	$0.89 \pm 0.02$	$2.19 \pm 0.01$	Fe(II) high spin

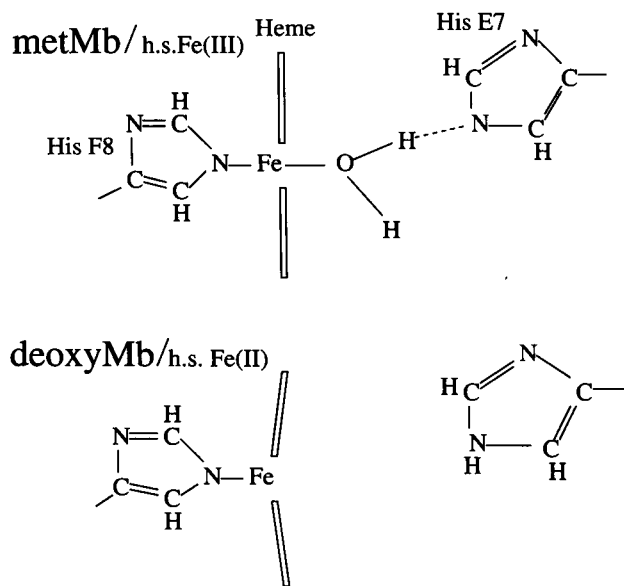


FIGURE 2 Environment of the iron in myoglobin. Distances in metMb (deoxyMb), respectively: Fe-heme, 0.2 Å (0.4 Å); Fe-93N<sub>2</sub>, 2.1 Å (2.1 Å); Fe-64N<sub>2</sub>, 4.4 Å (4.6 Å); Fe-O<sub>H<sub>2</sub>O</sub>, 2.2 Å.

radiation bind to deoxyMb molecules in the equilibrium conformation. The MbCO species are discussed later. The relative contributions of the observed iron species do not change for more than a month if the irradiated samples are kept in liquid nitrogen.

### Thermal cycling

Fig. 3 shows Mössbauer spectra of the irradiated metMb after

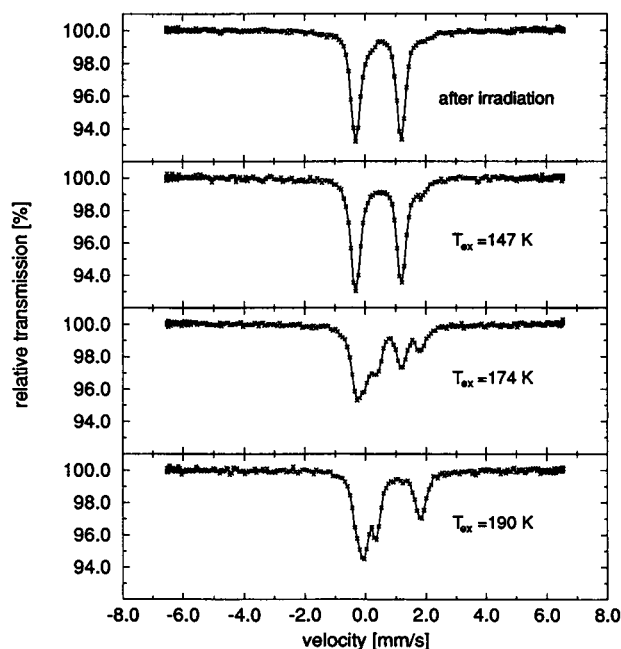


FIGURE 3 Mössbauer spectra of the irradiated metMb at  $T = 80$  K after different excursion temperatures,  $T_i$ . Incubation time, 45 min.

its warming up to a temperature,  $T_i$ , using the temperature cycling procedure described above. Fig. 4 gives the relative fractions  $P$  of the three iron states with the raise of the excursion temperature. The spectra indicate practically no changes up to 130 K. At 140 K the fraction of the Fe(II) low spin doublet begins to decrease, whereas that of the Fe(II) high spin increases. This tendency continues at higher excursion temperatures. Above 150 K the contribution of MbCO also begins to increase. It can be seen that the doublet of the nonequilibrium Fe(II) low spin completely disappears at  $\sim 200$  K.

The formation of MbCO indicates the presence of CO molecules in the sample. It is known that CO can be produced by the radiolysis of organic compounds, which contain  $=C=O$  and  $-C-O-$  groups (Farhataziz and Rodgers, 1987; Sharpatii, 1981). Glycerol with its three  $-COH$  groups per molecule can be an effective source of CO. The protein molecule also has CO groups, most of them close to the peptide bonds. However, the fraction of the protein CO groups is less than 5% of their total content in the sample. Therefore, the radiolysis of glycerol seems to be the main source of the CO molecules in our case. To test the possible influence of the solvent on the relaxation and on the CO production, we have also performed an experiment with low temperature irradiation of metMb crystals in the aqueous solution of  $(NH_4)_2SO_4$ . It has been found that the relaxation of the intermediate Fe(II) low spin complex begins and ends practically at the same temperatures ( $\sim 140$  and  $200$  K, respectively) as in the case of the water/glycerol solvent. The reduction of the heme Fe(III) in the sample without glycerol, however, is less effective and does not exceed 70–80% at the same irradiation dose. The observed Mössbauer spectra of the irradiated samples, therefore, contain the unresolved subspectrum of the initial Fe(III). Without glycerol the MbCO formation was not observed.

Our results show that the structural relaxation of the intermediate Fe(II) low spin molecules essentially starts at  $\sim 140$  K and is completed at  $\sim 200$  K. Above 150 K, the protein flexibility is enhanced enough to allow the movement of the water and CO molecules, respectively, from and into the heme pocket.

It should be noted that the Mössbauer hyperfine parameters of the produced iron species change slightly with the

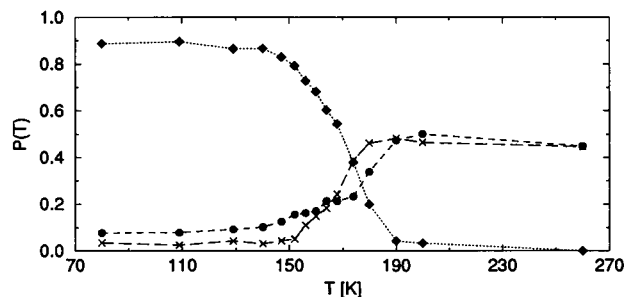


FIGURE 4 Relative fraction,  $P$ , of the observed iron states in the irradiated sample after different excursion temperatures,  $T_i$ : (♦) Fe(II) low spin; (●) Fe(II) high spin; (+) MbCO.

increase of the excursion temperature,  $T_i$ , although the Mössbauer spectra were always measured at 80 K. The greatest changes are in the case of the Fe(II) high spin, that is the product of the relaxation. This is not surprising because the Mössbauer parameters of these species are rather sensitive to alterations of the near surrounding. Fig. 5 shows that the isomer shift,  $IS$ , of the high spin Fe(II) is  $\sim 1.1$  mm/s immediately after the x irradiation and decreases with  $T_i$ , reaching the value of  $\sim 0.9$  mm/s above 170 K, which is typical for deoxyMb samples.

A first attempt to describe the structural relaxation obtained from thermal cycling experiments is given in Parak and Prusakov (1994). In that study, we used a Gaussian barrier height distribution. Further, we assumed that there exists a threshold barrier  $H_T$  for each excursion temperature that can be overcome within the experimental time (45 min in our case). All protein molecules in substates with barriers lower than  $H_T$  relax, whereas those with  $H > H_T$  remain in the intermediate state. The measurements of the kinetics, however, show that the relaxation essentially lasts longer and, hence, a better approach is needed.

## Kinetics

We used two identical samples for the study of kinetics, one sample for the measurements at 147 and 174 K and another one at 180, 190, and 195 K. Fig. 6 shows the time dependence of the relative fractions  $P$  (in logarithmic scale) of the intermediate Fe(II) low spin species. The relaxation into the Fe(II) high spin species is essentially nonexponential in time at all temperatures. It is not determined by a single enthalpy barrier of a unique height  $H$ . The strong decrease in  $P$  lasts longer (2–4 h) than the cycling time (45 min) used in our temperature studies of the relaxation. However, after some hours the kinetics of relaxation is strongly slowed down.

In analogy with the MbCO rebinding, we describe the relaxation of the intermediate Fe(II) low spin molecules into the equilibrium deoxyMb conformation assuming a distribution of activation enthalpies  $g(H)$ . The fraction of the unrelaxed molecules,  $N(t)$ , is then given by

$$N(t) = \int g(H) \exp[-k(H)t] dH \quad (1)$$

where  $k(H)$  is the rate coefficient for the relaxation.

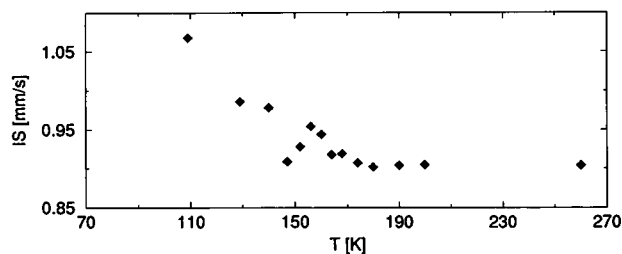


FIGURE 5 Isomer shift,  $IS$ , for the Fe(II) high spin component as a function of the excursion temperature,  $T_i$ .

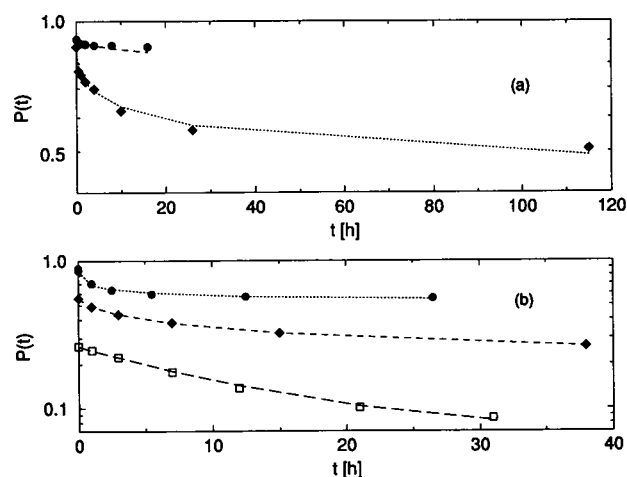


FIGURE 6 The relaxation kinetics of the intermediate Fe(II) low spin state in  $P$  (logarithmic scale) vs.  $t$  plot: (a) at 147 K (●) and 174 K (◆), (b) at 180 K (●), 190 K (◆), and 195 K (□).

Young and Bowne (1984) have proposed a gamma distribution of the barrier heights that was derived from a model of protein conformational substates:

$$g(H) = \frac{\alpha^{3\nu}}{\Gamma(3\nu)} (H - H_{\min})^{3\nu-1} \exp[-\alpha(H - H_{\min})], \quad (2)$$

where  $\Gamma(3\nu)$  is the gamma function.

The gamma distribution with an Arrhenius relation for the rate coefficient  $k(H, T) = A \exp(-H/(RT))$  gave good fits of the MbCO-rebinding kinetics up to 160 K. Recently, it has been shown that the nonexponential rebinding kinetics of NO in azurin can be explained by a Gaussian barrier height distribution (Nienhaus and Ehrenstein, 1992). Furthermore, it has been proven that the MbCO has at least four major, structurally different taxonomic substates (Ansari et al., 1987; Oldfield and Guo, 1991; Quillin et al., 1992). Consequently, the whole rebinding process probably could be described by a sum of four enthalpy distributions. Together with the result obtained in azurin, it is very likely that the gamma function-like distribution is actually a superposition of some Gaussians. One can expect that the metMb and the deoxyMb molecules also have more than one taxonomic substate contributing to the enthalpy distribution of the relaxation of the nonequilibrium Fe(II) low spin molecules. One of these substates can explain the relaxation of  $\sim 10\%$  molecules already occurring at 77 K. Nevertheless, a fit of the relaxation kinetics with one gamma distribution of the barrier heights is probably still a good approach.

Fig. 7 shows the fit for the relaxation kinetics at 147 and 174 K and the corresponding barrier height distributions. It must be noted that parameter  $A$  is taken to be  $10^8 \text{ s}^{-1}$ . Good fits of the experimental data can be obtained over a very large range of  $A$  values ( $10^4 \dots 10^{18} \text{ s}^{-1}$ ). The alteration of  $A$  correlates with some change of the  $H_{\min}$  value. An increase of  $A$  shifts the distribution,  $g_0$ , to slightly higher values, although the characteristic shape of the distribution is essentially conserved. The change of the enthalpy distribution in

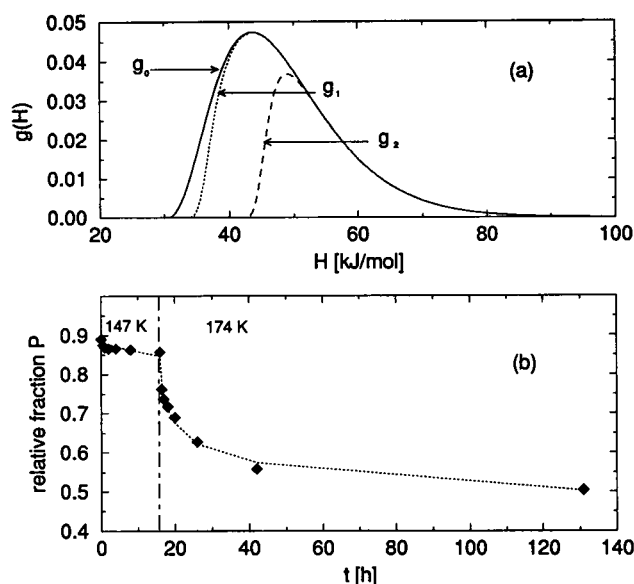


FIGURE 7 The fit of the relaxation kinetics at 147 and 174 K (b) by the gamma distribution according to Eq. 2 ( $A = 10^8 \text{ s}^{-1}$ ) and the relevant enthalpy distributions at the starting and ending time (a).

time (Fig. 7 a) reflects the sharp slowing down of the relaxation. The intermediates with lower activation enthalpy relax rather fast, but the rest practically cannot overcome their higher barriers at the given temperature.

The simultaneous fit of the kinetics at 147, 174 (sample 1), and 180 K (sample 2) describes the experimental data satisfactorily with a nearly identical initial distribution of the barrier heights. However, we failed to get a good fit of the relaxation process at all temperatures together, including the data at 190 and 195 K with one and the same initial barrier height distribution. Moreover, it proved to be impossible to fit the kinetics at only two temperatures (180 and 190 K, or 190 and 195 K) with one barrier height distribution (compare dotted lines in Fig. 8). These results show that the barrier

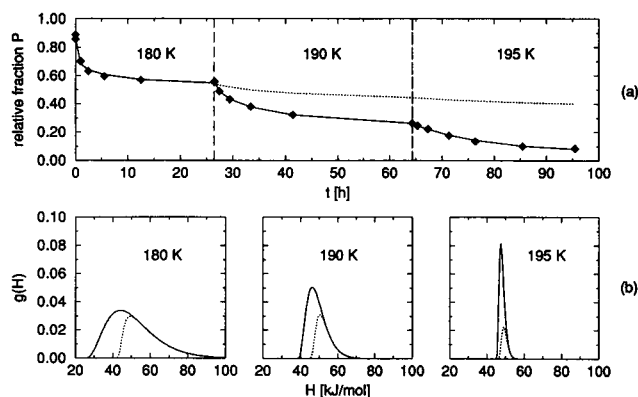


FIGURE 8 (a) The experimental data and the fit (—) of the relaxation kinetics at 180, 190, and 195 K by gamma distribution ( $A = 10^8 \text{ s}^{-1}$ ) assuming independent initial distributions for each temperature. The dotted line shows calculations of the kinetics at 190 and 195 K using the initial enthalpy distribution obtained at 180 K. (b) Distributions at the starting (—) and ending (·····) time yielding the solid lines in a.

height distribution is a function of temperature above 180 K. The kinetics at 190 and 195 K was fitted independently, normalizing the corresponding initial distribution for the given temperature to the final enthalpy distribution at the preceding temperature. Fig. 8 gives the results and the obtained enthalpy distributions at the beginning and the end of each relaxation experiment. It is seen clearly that with increasing temperature the initial barrier height distribution becomes narrower and the peak value of the enthalpy,  $H_{\text{peak}}$ , decreases in comparison with the value at the end of the relaxation experiment of the preceding temperature. Although below 180 K the barrier height distribution is changed with time only by depopulation at the low energy side, above 180 K the barrier height distribution by itself becomes a function of temperature and time. This reflects rearrangements of the myoglobin structure above 180 K. They become possible because of the dramatic enhancement of the protein flexibility in this temperature range seen, e.g., in Mössbauer experiments (Frauenfelder et al., 1988; Parak and Formanek, 1971; Parak et al., 1981; Parak et al., 1982; Bauminger et al., 1983; Parak and Frauenfelder, 1993).

To understand the experimental results, one can start from a hierarchical order of dynamical processes in proteins (Ansari et al., 1985). Although local dynamics can occur already at low temperatures, higher temperatures are necessary for structural fluctuations involving larger parts of the molecules and going over larger distances. In this picture, the temperature around 180 K may be a border between two hierarchies. Although below 180 K only local rearrangements are possible, global structural relaxations can occur at higher temperatures. The relaxation of the Fe(II) low spin intermediate to the Fe(II) high spin state needs removal of the water molecule coordinated to the iron. This process depends not only on the strength of the bond itself and the relative position of the heme iron but, more likely, on the steric hindrance in the protein that prevents the release of the water molecule. It is known that the coordinated water molecule in metMb is hydrogen-bonded to the distal E7 histidine and is in van der Waals contact with the methyl group of the valine E11 (Petrut, 1979). A shift of these residues can affect the kinetics of the relaxation process Fe(II) low spin to Fe(II) high spin. Conformational substates coming from a structural distribution of the HIS E7 and the VAL E11 may contribute substantially to the barrier height distribution that governs the relaxation kinetics below 180 K. It is reasonable to assume that below 180 K relaxation from the met to the deoxy conformation occurs only partly. It allows, however, the water molecule to separate far enough from the iron to decrease the crystal field so that the iron becomes Fe(II) high spin. Nevertheless, the water remains in the heme pocket.

The situation changes above 180 K. Now the protein molecule is flexible enough to perform larger structural rearrangements. The obtained enthalpy distributions for the relaxation are effective ones. They are determined by many protein coordinates and can be conditionally described through a generalized protein coordinate (Agmond and Hopfield, 1983). Formally, we can describe the temperature and

time dependence of the change of the  $H_{\text{peak}}$  by a relaxation function  $\phi^*(t, T)$  (Berlin et al., 1992):

$$H_{\text{peak}}(t, T) = H_{\text{peak0}} + H^*[1 - \phi^*(t, T)]. \quad (3)$$

Without additional information on the physical nature of the enthalpy shift and the narrowing of the distribution, we believe that such a discussion should be postponed.

It is obvious that the described r-t transition shows similarities with the deoxyMb-MbCO relaxation investigated intensively by the CO-rebinding kinetics after the flash photolysis of the CO. However, we would like to stress that one cannot simply understand both phenomena with the identical physical picture. Below 180 K the CO molecule flash-photolyzed from the heme iron remains in the heme pocket and cannot diffuse out of the myoglobin molecule (Steinbach et al., 1991). Above 160 K the barrier height distribution depends on temperature. These features can be explained by two processes: a relaxation along a reaction coordinate and a conformational relaxation of the protein molecule. Below 160 K the rebinding of CO along the reaction coordinate reflects the structural distributions in the ensemble of molecules by a distribution of enthalpic barriers. Conformational relaxations move this distribution to higher energies above 160 K. Above 180 K conformational relaxations come in on a different tier narrowing the energy distribution and decreasing the average barrier height. In the case of the metMb-deoxyMb transition, it is difficult to define a reaction coordinate. When the iron is reduced and the molecule is in the Fe(II) low spin state, it is still unclear whether the low spin state is stabilized mainly by the conformation of the protein or by the 6th coordination of the iron with the water molecule. In contrast with the CO molecule, the water never coordinates again with the iron after local relaxations released it into the heme pocket. A strong analogy between metMb-deoxyMb and MbCO-deoxyMb transitions probably exists mainly at temperatures above 180 K. In both cases, conformational relaxations narrow the barrier distributions and decrease the average barrier height.

Some additional information on the relaxation process of the metastable Fe(II) low spin conformation follows from the temperature and time dependencies of the hyperfine parameters of the Fe(II) high spin species that belongs to the final equilibrium conformation. Figs. 5 and 9 show that the values of  $IS$  and  $QS$  immediately after x-ray irradiation differ from those of equilibrium deoxyMb and begin to change already at the lowest studied temperature of 109 K. At 147, 174, and 180 K, their alteration continues for several hours. This can be understood from the comparison of the structures of the protein active center in metMb and deoxyMb (Fig. 2). In metMb the heme is essentially planar and the iron atom is displaced by only 0.2 Å from the heme toward the proximal imidazole. In deoxyMb the heme is domed and the iron-heme distance is  $\sim 0.4$  Å. One can suppose that the  $IS$  and  $QS$  values observed immediately after the low temperature irradiation of the samples correspond to the Fe(II) high spin, where the surrounding is closer to the initial (metMb) geometry of the active center. The different hyperfine param-

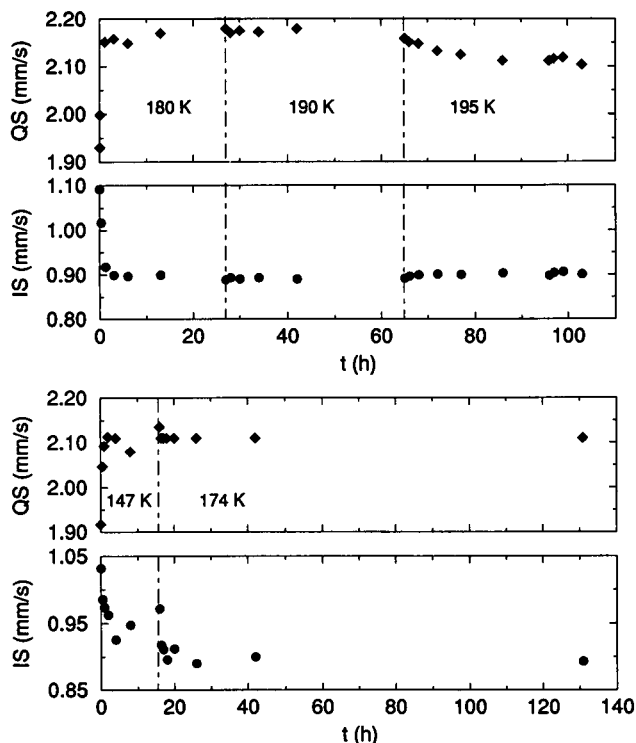


FIGURE 9 Isomer shift,  $IS$ , and quadrupole splitting,  $QS$ , for the Fe(II) high spin component as a function of the time in the kinetics experiments.

eters represent a distribution of conformational substates of the Fe(II) high spin myoglobin, which is broader than in equilibrated deoxyMb. One can assume that the substates that are more similar to the metMb structure are populated first. Increasing temperature allows gradual alterations of the geometry toward the equilibrium deoxyMb conformation.

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