A Physical Picture of Protein Dynamics and Conformational Changes

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Abstract A physical model is reviewed which explains different aspects of protein dynamics consistently. At low temperatures, the molecules are frozen in conformational substates. Their average energy is $3/2RT$. Solid-state vibrations occur on a time scale of femtoseconds to nanoseconds. Above a characteristic temperature, often called the dynamical transition temperature, slow modes of motions can be observed occurring on a time scale between about 140 and 1 ns. These motions are overdamped, quasidiffusive, and involve collective motions of segments of the size of an α -helix. Molecules performing these types of motion are in the “flexible state”. This state is reached by thermal activation. It is shown that these motions are essential for conformational relaxation. Based on this picture, a new approach is proposed to understand conformational changes. It connects structural fluctuations and conformational transitions.

Keywords Protein dynamics · Conformational relaxation · Mössbauer effect · Myoglobin

1 Introduction

The function of a protein is strongly correlated with its structure and its dynamics. Since the pioneering work of Hans Frauenfelder [1, 2], protein dynamics has been at the center of interest of many biophysical groups. The field of protein dynamics has many aspects. At

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present, greatest attention is given to the problem of protein folding. Large-scale motions are necessary to come from the polypeptide chain into the functional active structure of a protein molecule. But, also in the functional well-folded state, significant structural changes occur. Proteins which bind small ligands have normally two different conformational structures, a conformation with the bound ligand and one with empty binding position. A well-known example is the tensed (t) and the relaxed (r) conformation of myoglobin and hemoglobin. Both proteins bind oxygen or other small molecules like CO on the heme iron buried within the interior of the molecule. While the ligated molecules are in the r conformation, the molecules without a ligand are in the t conformation [3]. The conformational transition requires structural rearrangements within the molecules. It was shown that diffusion-like motions of molecular segments are the lubricant which makes conformational transitions possible [4]. Besides these protein-specific aspects, one should not forget that proteins exhibit the physics of condensed matter. This means that optical and acoustic phonons are present. There is one further peculiarity of protein molecules. Even molecules in one and the same conformation (e.g., t conformation, which means no ligand at the heme iron) differ slightly in their structure, having nevertheless practically the same energy. It is common to call these slightly different structures, which in general cannot be resolved in X-ray structure analysis, conformational substates [5]. This feature is often called “glass-like behavior” of proteins.

In this contribution, we want to review a special physical description of several of the features mentioned above, not discussing, however, the folding problem. The picture is developed using experiments performed on sperm whale myoglobin [6, 7]. This α -helical O₂-storing protein with a molecular mass of 17,000 Da is one of the best-studied proteins. Based on the review, we outline a new way to understand conformational changes. This approach correlates structural fluctuations and conformational relaxation.

2 X-ray Structure Determination of Mean Square Displacements

Besides the coordinates of all nonhydrogen atoms, the X-ray structure analysis of protein crystals yields the mean square displacements, $\langle x^2 \rangle^X$, of the individual atoms in the molecule from the Debye-Waller factor. The first analyses along these lines were published in the classical papers of Frauenfelder, Petsko, and Tsernoglou [8] and by Artymiuk et al. [9]. In [8], it was tried to get the potentials determining the $\langle x^2 \rangle^X$ values from their temperature dependence. In spite of the fundamental character of this publication, the paper had two shortcomings. First of all, the investigated temperature regime was relatively small. Later experiments including temperatures down to 140 K showed that most of the mean square displacements decreased linearly with temperature. An extrapolation of this behavior to $T = 0$ K showed that besides the zero point vibrations there remained a static disorder [10]. The second shortcoming was more difficult to be removed. The mean square displacements obtained from the Debye-Waller factor are a sum of intra- and inter- molecular displacements. In other words, TLS (translation, libration, screw) - modes contribute as well to the $\langle x^2 \rangle^X$ values as displacements within the molecules. This problem was solved by the normal mode refinement developed by Go and Kidera [11, 12] and later applied to myoglobin [13]. In the following analysis, we assume that the static disorder obtained from the extrapolation of the $\langle x^2 \rangle^X$ values to $T = 0$ K does not influence their temperature dependence. Furthermore, our picture has to yield the linear temperature dependence of individual mean square displacements as an ensemble average, in agreement with the X-ray structure analysis.

The mean square displacements obtained from X-ray structure analysis are described by:

$$\langle x^2 \rangle^x = \langle x_{\text{stat}}^2 \rangle^x + \text{const} \cdot T \quad (1)$$

where the upper index X refers to X-ray measurement and the lower index stat to the part which remains at the linear extrapolation to the temperature $T = 0$ K. All mean square displacements are ensemble averages. The potential energy $W[T]$ is distributed according to Maxwell-Boltzmann replacing the kinetic energy by the potential energy $k_H \langle x^2 \rangle / 2$. Here, k_H is a spring constant according to Hooke's law. $\langle W[T] \rangle = 3RT/2$ is the average value. R equals 8.314 J/(mol K).

3 Phonon-Assisted Mössbauer Scattering

The dynamic properties of solids are described by phonons. The use of synchrotron radiation allows the determination of the density of phonon states in proteins. Especially, phonons which couple to an iron atom can now be measured. The method is based on the fact that the Mössbauer isotope ^{57}Fe has a nuclear transition of 14.4 keV. Incoming radiation which matches this energy within a width of 10^{-9} eV is scattered with a time delay corresponding to the mean lifetime of this level of 140 ns. This fact allows the separation of the nuclear resonant scattered radiation from the Rayleigh scattering by the electrons of the sample which occurs without time delay [14–17].

Figure 1 shows the experimental setup. With the help of a sophisticated monochromator system a beam with energy of about 14.4 keV and a width of about 0.85 meV is separated from the broad spectrum of the undulator U. Due to the bunch structure of electrons in the synchrotron, the beam is pulsed. The sample is irradiated for 176 ns by X-ray bunches with a length of about 100 ps. Fast detectors count the scattered radiation. They are gated during the prompt Rayleigh scattering and active only during the time between the X-ray pulses.

Fig. 1 Experimental setup for phonon-assisted Mössbauer effect. All 176 ns bunches of X-rays with a length of 100 ps and an energy of about 14.4 keV are produced by an undulator U and hit the sample. Two monochromator systems, a Si(111) premonochromator and a Ge(331)/Si(975) high resolution monochromator, reduce the energy width to typically 0.85 meV. They allow a variation of the energy of ± 80 meV around the resonance energy of the ^{57}Fe Mössbauer transition. The scattered radiation is collected by avalanche photo detectors APD1a and APD1b. The energy resolution is measured by forward scattering into APD2. The counters are gated during the time when the X-ray bunch hits the sample

In this way, they measure only the time-delayed nuclear resonant scattering (Mössbauer scattering). One may now adjust the monochromator system so that the X-ray energy is too low (or too high) to excite the nuclear resonance transition. We term the energy mismatch ε . Nevertheless, nuclear resonance scattering occurs. The missing energy ε is provided by phonons of the energy ε . In this way, the intensity of the nuclear resonance scattering becomes a measure of the number of phonons with energy ε . Of course, phonons can also take up energy if the incoming radiation is too high by an energy ε . Varying the mismatch energy ε we determined the phonon density spectrum $D(\varepsilon)$ from 0 to 70 meV. Figure 2 shows the density of phonon states coupling to the heme iron of metmyoglobin (Mbmet) [17, 18]. Having $D(\varepsilon)$ it is easy to calculate the mean square displacements at the iron due to a phonon of energy ε , according to [19]:

$$\langle x^2(\varepsilon) \rangle^{\text{phon}} = \frac{\hbar^2}{m_{\text{Fe}}} \cdot \frac{D(\varepsilon)}{\varepsilon} \cdot \left(\frac{1}{\exp\left(\frac{\varepsilon}{k_B T}\right) - 1} + \frac{1}{2} \right), \quad (2)$$

where $\langle x^2(\varepsilon) \rangle^{\text{phon}}$ is the mean square displacement of the heme iron stemming from phonons of the energy ε . The mass of the iron is denoted m_{Fe} . $\hbar$ and k_B are the Planck and the Boltzmann constant, respectively. For details, compare [17]. For a comparison with other data, $\langle x^2(\varepsilon) \rangle^{\text{phon}}$ was integrated over ε from 0 to 70 meV yielding $\langle x^2 \rangle^{\text{phon}}$. Measurements between 50 and 300 K showed a linear increase of $\langle x^2 \rangle^{\text{phon}}$ with temperature and some small inharmonicity at room temperature. Figure 3 shows, in addition, the temperature dependence of $\langle x^2 \rangle^{\text{NM}}$ values obtained from a normal mode analysis [20]. It is obvious that a normal mode analysis can explain the $\langle x^2 \rangle^{\text{phon}}$ values. For a detailed discussion of this finding, we refer to [17].

For further discussion, it is important to look on the time scale of the vibrations. It is determined by the energy resolution, ΔE , of the experiment.

$$\Delta E \cdot \Delta t = \hbar, \quad \Delta t = 1/\Delta\omega, \quad (3)$$

where Δt is a time resolution which can also be expressed as the frequency resolution $\Delta\omega$. In the present case, the energy width of about 0.85 meV of the monochromator of the

Fig. 2 Density of states of phonons coupling to the heme iron in Mbmet. It was calculated from experiments at 50, 170, 235, and 300 K

Fig. 3 **a** A comparison of the temperature dependence of mean square displacement values determined by different techniques. *Diamonds and thick solid line:* $\langle x^2 \rangle^{\text{phon}}$ phonon-assisted Mössbauer effect; *dashed line:* $\langle x^2 \rangle^{\text{NM}}$ values obtained from a normal mode analysis; *circles:* $\langle x^2 \rangle^{\gamma}$ values obtained from Mössbauer absorption spectroscopy on the heme iron of Mbmet; *thin solid line:* least squares fit using the Brownian oscillator model with $\Delta H_{\text{flex}} = 35,000$ J/mol and $\Delta S_{\text{flex}} = 121.3$ J/K/mol. **b** and **c** X-ray structures of the L29W mutant of MbCO at 105 K [4]. In both figures, the dark structure of MbCO is shown as model. The electron density represents the structure after photolysis of the CO at 105 K (**b**) and above 180 K (**c**). Panels **b** and **c** are reprinted by permission from Macmillan Publishers Ltd, *Nature* **404**, 205–208, 2000

synchrotron radiation yields a time threshold of about 0.65 ps. Only vibrations faster than 0.65 ps contribute significantly to the mean square displacements.

4 Slow Motions Investigated by Mössbauer Absorption Spectroscopy

If we want to see slower motions, we have to increase the energy resolution of the experiments. Mössbauer spectroscopy has a very high energy resolution. In case of the isotope ^{57}Fe , the bandwidth $\Delta E = \Gamma_{\text{nat}}$ becomes $4.7 \cdot 10^{-9}$ eV. This corresponds to a time threshold of 140 ns. The shape of the energy resolution function is a Lorentzian. Moreover, Mössbauer spectroscopy is a very good tool to determine mean square displacements, $\langle x^2 \rangle^{\gamma}$ of the heme iron from the Lamb-Mössbauer factor f [21–23]:

$$f = \exp \left(-k_{\text{Fe}}^2 \langle x^2 \rangle^{\gamma} \right). \quad (4)$$

Here, $k_{\text{Fe}} = 2\pi/\lambda$ with $\lambda = 0.86$ Å for ^{57}Fe . The Lamb-Mössbauer factor can easily be determined from the area of the Lorentzians which fit the absorption spectrum. For a review of the Mössbauer effect, see [24]. All motions faster than 140 ns, which couple to the iron, contribute to the mean square displacement. Figure 3 shows $\langle x^2 \rangle^{\gamma}$ values obtained from Mössbauer absorption spectroscopy. One can distinguish two temperature regions. Below a characteristic temperature, T_c , often called the dynamic transition temperature, the mean square displacements increase linearly with temperature [23]. A normal mode

analysis yields the same values [20]. Moreover, the slope of $\langle x^2 \rangle^{\text{phon}}$ with temperature obtained from phonon-assisted Mössbauer experiments coincides with the slope of $\langle x^2 \rangle^\gamma$ [17]. The conclusion is that Mössbauer spectroscopy below T_c measures essentially fast vibrations as $\langle x^2 \rangle^{\text{phon}}$ and $\langle x^2 \rangle^{\text{NM}}$. $\langle x^2 \rangle^\gamma$ equals $\langle x_{\text{vib}}^2 \rangle$ where the index *vib* stands for vibrations. The picture changes above T_c . The rapid increase of the $\langle x^2 \rangle^\gamma$ values shows that another type of motions contributes here, yielding $\langle x_{\text{flex}}^2 \rangle^\gamma$. For an understanding of the physical nature of these motions, one has to consider the temperature dependence of the shape of the Mössbauer spectra. While below T_c the Mössbauer spectra can be fitted by Lorentzians with common line widths, additional broad lines are necessary above T_c [25, 26]. Such broad lines indicate diffusive motions in a limited space and are often called quasidiffusive. An analysis of the broad lines yields information on the characteristic times of the quasidiffusive motions. An example is given in Fig. 4. The x-axis of the spectrum gives the energy in units of the Doppler velocity, v , which is the velocity of the radiation source with respect to the sample. The energy shift from the resonance energy equals $v/cE\gamma$, where $E\gamma$ is about 14.4 keV and c is the velocity of light. The characteristic time is obtained from Eq. 3. Figure 4 shows that the absorption of the broad line indicates a sensitivity for times between 140 ns and about 1 ns. The long time limit is given by the natural life time of the ^{57}Fe nucleus. For times faster than about 1 ns, the absorption becomes immeasurable. The described time sensitivity for quasidiffusive processes should not be confused with the time sensitivity of the Lamb-Mössbauer factor which has only a border at 140 ns. All motions faster than 140 ns contribute to $\langle x^2 \rangle^\gamma$. Taking both temperature regions together, we have:

$$\langle x^2 \rangle^\gamma = \langle x_{\text{vib}}^2 \rangle^\gamma + \langle x_{\text{flex}}^2 \rangle^\gamma. \quad (5)$$

The $\langle x_{\text{flex}}^2 \rangle^\gamma$ contribution approaches zero below T_c .

For an understanding of all features discussed up to this point, a two-state model has been developed [23]. At low temperatures ($T < T_c$), the molecules are trapped in a conformational substate (CS). The molecules have slightly different structures and perform solid-state vibrations as determined by phonon-assisted Mössbauer spectroscopy. There is no difference, in principle, from a solid. With rising temperatures, new modes of motions contribute and become measurable above T_c causing $\langle x_{\text{flex}}^2 \rangle^\gamma$ and the broad lines. As shown

Fig. 4 Mössbauer spectrum of Mbmet crystals at 295 K. The energy scale of the x-axis is given in Doppler velocities. These units can be converted into characteristic times of motions

in several publications [25–27], this behavior can be understood by introducing a second state, the flexible state. The temperature-dependent probability, $p_F[T]$, that a molecule is in the flexible state F is given by:

$$p_F[T] = \left(1 + e^{(\Delta G_{\text{flex}}/R/T)}\right)^{-1}. \quad (6)$$

$\Delta G_{\text{flex}} = \Delta H_{\text{flex}} - T\Delta S_{\text{flex}}$ is the free Gibbs energy difference between a CS and the flexible state. The motions in the flexible state have been described by a Brownian oscillator using the Langevin equation plus a restoring force [23]:

$$\ddot{x}(t) + 2\beta\dot{x}(t) + \omega^2 x(t) = F(t). \quad (7)$$

A stochastic force $F(t)$ produces an acceleration along the x direction which is damped with a damping constant β . The restoring force is taken into account by ω . Note that this force is assumed to be harmonic in the simplest approach. Actually, the motion is three-dimensional, and x is the component parallel to the wave vector k_{Fe} of the γ -beam of the spectrometer. At this point, we want to emphasize that the diffusive motions are not explained by a hopping model, where the molecule jumps between local minima. Such a model was not suitable to explain the temperature dependence of the Mössbauer spectra [28]. Here, a classical picture is used. The molecule is for a real time in the flexible state.

The Mössbauer absorption cross section is then given by [23]:

$$\sigma_a(E_a, E) = \sigma_0 \frac{\Gamma_{\text{nat}}}{2} e^{-k_{\text{Fe}}^2 \langle x_{\text{vib}}^2 \rangle^\gamma} e^{-k_{\text{Fe}}^2 \langle x_{\text{flex}}^2 \rangle^\gamma} \sum_{N=0}^{\infty} \frac{[k_{\text{Fe}}^2 \langle x_{\text{flex}}^2 \rangle^\gamma]^N}{N!} \frac{(\frac{\Gamma_a}{2} + N\hbar\alpha)}{(E - E_a)^2 + (\frac{\Gamma_a}{2} + N\hbar\alpha)^2}. \quad (8)$$

E_a is the resonance energy of the iron. σ_0 equals $2.56 \cdot 10^{-18} \text{ cm}^2$. Γ_{nat} is the natural line width of $4.7 \cdot 10^{-9} \text{ eV}$ of the 14.4 keV level of ^{57}Fe , determined by its mean life time $\tau_N = 140 \text{ ns}$. Γ_a gives the experimental line width of the heme iron, which may be larger than Γ_{nat} . Besides $\langle x_{\text{vib}}^2 \rangle^\gamma$ and $\langle x_{\text{flex}}^2 \rangle^\gamma$ the fit of the Mössbauer spectra with Eq. 8 yields the parameter

$$\alpha = \frac{\omega^2}{2\beta} \quad \text{which equals} \quad p_F(T) \alpha_0 \quad (9)$$

where $p_F(T)$ (Eq. 6) has to be determined from the temperature dependence of $\langle x_{\text{flex}}^2 \rangle^\gamma$ using:

$$\langle x^2 \rangle^\gamma = \langle x_{\text{vib}}^2 \rangle^\gamma + p_F(T) \langle x_0^2 \rangle^\gamma \quad \text{with} \quad \langle x_0^2 \rangle^\gamma = c_{\text{flex}} \cdot T. \quad (10)$$

According to Eqs. 6 and 10, three parameters have to be fitted, ΔH_{flex} , ΔS_{flex} , and c_{flex} .

Figure 3a shows a fit of the $\langle x^2 \rangle^\gamma$ values in the temperature range from 80 to 340 K, yielding $\Delta H_{\text{flex}} = 35,000 \text{ J/mol}$ and $\Delta S_{\text{flex}} = 121.3 \text{ J/K/mol}$. The parameter α_0 was taken to be $6.64 \cdot 10^8 \text{ s}^{-1}$, in consistence with [29] for MbCO. The described picture is certainly a strong simplification. There is not only one well-defined CS but several. There may be several flexible states with slightly different parameters. However, the successful analysis of the experimental data shows that only two effective states are a good approach.

It should be mentioned in passing that incoherent neutron scattering revealed a similar picture for the dynamics of the hydrogens. Details can be found in [30] and [31].

5 The Size of Collectively Moving Segments

At this point of the review, the type of motion in the flexible state is explained. However, it is still unclear what “units” perform the Brownian oscillations. Since the experiments have been performed on crystals, it is unlikely that the protein molecules, as a whole, perform diffusive motions. However, the motions have to be highly correlated, which means that parts of the molecule have to be involved. To estimate the size of the moving parts, another variant of Mössbauer spectroscopy has been used. The Rayleigh Scattering of Mössbauer Radiation (RSMR) is a technique which allows to measure slow motions in a sample without a Mössbauer isotope. For a review of the method, see [32, 33] and for the application on biomolecules, [34–37]. In the experiment, we refer to [38] where myoglobin with the natural mixture of Fe isotopes has been used. The 2.2% of ^{57}Fe could be neglected. The principle of the experiment is shown in Fig. 5. A Mössbauer source S , usually ^{57}Co which decays to ^{57}Fe , is mounted on a driving system. The source emits 14.4 keV radiation with $\Gamma_{\text{nat}} = 4.7 \cdot 10^{-9}$ eV. As in the conventional Mössbauer experiments, its energy is varied by the Doppler Effect. The radiation is scattered by the sample P . The scattered radiation is collected at well-defined angles. For an energy analysis, a Mössbauer absorber is used. With this analyzer in the direct beam (A0 in Fig. 5), the intensity I_0 of the radiation from the source is determined, which can perform Mössbauer absorption in the analyzer. With the analyzer in the scattered beam (A2 ϑ in Fig. 5), the part I_{scatt} was determined which can still be absorbed by the Mössbauer effect. I_{scatt} is normally smaller than I_0 . Part of the elastic-emitted radiation of the source is inelastically scattered and can, therefore, no longer be absorbed by nuclear resonance. Due to the small Γ_{nat} , scattering involving motions on a time scale faster than about 140 ns are measured as inelastic. The result is shown in Fig. 6. The angular dependence of I_{scatt} allows us to determine the size of the moving segments responsible for the inelastic scattering. While at $\sin\vartheta/\lambda = 0.05 \text{ \AA}^{-1}$ the resolution is about 10 Å, and it decreases about 3.3 Å at $\sin\vartheta/\lambda = 0.15 \text{ \AA}^{-1}$. At small scattering angles, collectively moving segments become important. In Fig. 6, the experimental data are compared with three different models. In the first model, all atoms of the molecule move independently. In the second, segments of the size of α -helices move collectively, while in the third model, the whole molecule performs collective motions. It is obvious that at large angles, the models coincide as expected. Collective motions become significant only at small scattering angles. The experimental data are best fitted by the model of collectively moving segments of the size of an α -helix. Note that the model of collectively moving total molecules does not fit at all to the experiments.

Fig. 5 Experimental setup for a RSMR experiment. S : Mössbauer source mounted on a Doppler drive; $A0$ and $A2\vartheta$: Mössbauer absorber either in direct beam or in scattering direction, respectively; P : protein sample; D : detector

Fig. 6 RSMR: *Crosses*: inelastic scattering as function of scattering angle; *dashed, solid, and dashed dotted lines*: models for individually moving atoms, collectively moving segments of the molecule with the size of an α -helix and collective motion of the whole molecule, respectively [38]

6 The Functional Relevance of Slow Quasidiffusive Motions

After determining motions within a protein with characteristic times from picoseconds to at least 140 ns, there remains the question of functional relevance. As mentioned in Section 1, myoglobin makes an $r \rightarrow t$ transition if a ligand on the heme iron is removed. If such a conformational change is blocked, the protein has lost its functionality. This effect was investigated by structure determination of a myoglobin mutant L29W at different temperatures. First, a structure of the CO-ligated myoglobin was determined at 105 K in the dark. In this way, we obtained a reference structure of the ligated myoglobin. This structure was compared with two structures after photolysis of the CO ligand by laser light. Both structures were measured at 105 K. However, the photolysis was performed at 105 and above 180 K, respectively. Figure 3 shows the result. A photolysis below T_c does not change the structure. There is no conformational change. Only the dissociated CO molecule docks below the tryptophan in the heme pocket (Fig. 3b). However, a photolysis of CO above T_c results in a large conformational relaxation (Fig. 3c) [4]. The conformational relaxation occurs if slow collective motions are activated. Such diffusive motions may serve as a lubricant for the structural relaxations.

Besides the photolysis of CO, we used another method to trigger a conformational relaxation. In Mbmet, the heme iron is coordinated by a water molecule. The iron is in the Fe(III) high-spin state. An irradiation of the sample by X-rays produces electrons which can reduce the iron. At temperatures of 80 K, one obtains metastable molecules which still have the structure of Mbmet with water on the sixth coordination site (compare [39]). However, the iron is in the Fe(II) low spin state. We call it Mb(II,ls,w). Raising the temperature, the molecules relax into deoxymyoglobin with Fe(II) high spin and no ligand at the sixth coordination site, Mb(II,hs). Well below T_c obtained by Mössbauer spectroscopy, the structure is relatively stable, although a small structural relaxation occurs already at 147 K but on a time scale of many hours. The water molecule on the sixth coordination site can be photo-dissociated. However, it cannot leave the heme pocket and binds back. This process takes place on time scales between nanoseconds and seconds at temperatures from 60 to 150 K. For rebinding, the water has to surmount a distributed barrier quite similar to the CO-rebinding experiments (ΔH peaked at 8 kJ/mol, $\Delta S = -80.7$ J/mol/K). No structural relaxation occurs on the time scale of the experiment [40].

The fraction $n(t)$ of Mb(II,ls,w) which remained after the time t at the temperature T_{expl} was determined from Mössbauer spectra. After the reduction by X-ray irradiation at 80 K, a

Fig. 7 Relaxation of the metastable Mb(II,ls,w) (r conformation) into the equilibrium conformation Mbdeoxy (t conformation). y-axis: remaining fraction $n(t)$ of Mb(II,ls,w) after the time t . Sample 1, circles: Relaxation at 147 and 174 K; sample 2, diamonds: relaxation at 180, 190, and 195 K [41]. The temperature decreases from top to bottom. Lines: fits with the parameters given in Table 1. Solid line for 147 K, dashed line: 174 K, dotted line: 180 K, dashed dotted line: 190 K, dashed double dotted line: 195 K. Thin dashed and dotted lines for 174 and 180 K represent the time course without shift of the enthalpy according to Eq. 19

Mössbauer spectrum was taken. Then, the sample was warmed up to a certain temperature T_{expl} and kept at this temperature for some time t . Then, it was cooled down to 80 K where the Mössbauer spectrum was measured again yielding $n(t_1)$ at T_{expl} . The cycle warming up to T_{expl} , keeping the sample at this temperature for a time interval Δt , cooling down to 80 K, and measuring the Mössbauer spectrum was repeated several times. Two samples were investigated: one at T_{expl} of 147 and 174 K (sample 1) and another at 180, 190, and 195 K (sample 2). The experimental results are shown in Fig. 7. The experiments have been analyzed by a simple model where a distributed energy barrier had to be surmounted for structural relaxation [41].

It should be mentioned in passing that the artificial-looking intermediate Mb(II,ls,w) could well be of biological importance. Hemoglobin in the blood auto-oxidizes to a certain degree to methemoglobin with the iron in the Fe(III,hs,w) state. The enzyme methemoglobin reductase catalyzes the conversion of the iron back into the biological active II state. A Fe(II,ls,w) state is a necessary intermediate.

7 Proposal of a Model Connecting Conformational Relaxation and Structural Fluctuations

A molecule in a well-defined conformation has a certain probability to be in the flexible state. This should be valid also for molecules in a metastable state. It is, then, reasonable to assume that conformational transitions are mainly performed by molecules in the flexible state since the necessary ΔG_{rel} to reach a transition state is smaller than for molecules

in a CS. These assumptions could explain the correlation of conformational changes and structural fluctuations discussed in Section 6.

For a quantitative treatment, we start with the following reaction scheme:

where CS , F , and R represent conformational substates, the flexible state, and the relaxed state, respectively. R is identical with the new conformation after the conformational transition. cs , f , and r represent the number of molecules in the different states. It is assumed that the rates k_1 and k_{-1} are faster compared to k_2 . There is no back rate from R to F . Note that this is equivalent to the Michaelis-Menten kinetics in biochemistry [42]. As a consequence of the assumptions, we obtain:

$$\frac{f}{cs + f} = \frac{f}{n} = p_F(T). \quad (12)$$

This is identical to Eq. 6. At $t = 0$, there are no molecules relaxed, $n = n_0$. The number of molecules in the conformation, $r(t)$, increases with time while the still unrelaxed molecules $n(t)$ decrease:

$$\frac{dr(t)}{dt} = -\frac{dn(t)}{dt} = f \cdot k_2 = p_F(T) \cdot n(t) \cdot k_2 \quad (13)$$

which finally yields:

$$n(t) = n_0 e^{-p_F(T) \cdot k_2 t} \quad (14)$$

where $n(t)$ is determined by the experiment for different temperatures, and $p_F(T)$ can be taken from the Mössbauer experiments according to Eq. 6. The fit parameter is k_2 .

For a determination of k_2 , we remember that the motions in the flexible state can be described as overdamped Brownian oscillations (compare Eq. 7). This immediately implies that the transition to R has to be described by Kramers theory [43, 44]. Using the notation of Eqs. 6 and 9 and assuming the same frequency ω in the state F and at the top of the barrier which has to be surmounted to relax into R , we obtain:

$$k_2 = \frac{\alpha_0}{2\pi} e^{-\Delta G_{\text{rel}}/R/T} \quad (15)$$

where α_0 is taken from the Mössbauer experiments. ΔG_{rel} is the Gibbs free energy difference between the state F and the top of the barrier between F and R . As usual, $\Delta G_{\text{rel}} = \Delta H_{\text{rel}} - T\Delta S_{\text{rel}}$. A comparison with the experimental data shows that at low

Table 1 Parameters which describe the structural relaxation of Mb(II,ls,w) using Eqs. 16–19

	Sample 1	Sample 1	Sample 2	Sample 2	Sample 2
$T[\text{K}]$	147	174	180	190	195
$\Delta H_{\text{rel}0} [\text{kJ/mol}]$	34.7	34.7	36.3	41.9	42.3
$\Delta \Delta H_{\text{rel}0} [\text{kJ/mol}]$	—	3.5	3.5	—	—
β_{rel}	0.55	0.55	0.48	0.43	0.76
$\Delta S_{\text{rel}} [\text{J/mol/K}]$	14.8	14.8	14.8	14.8	14.8

temperatures, ΔH_{rel} has to be distributed. One exponential decay cannot explain the time course of $n(t)$, and k_2 becomes:

$$k_2 = \frac{\alpha_0}{2\pi} e^{\Delta S_{\text{rel}}/R} \int g(\Delta H_{\text{rel}}) e^{-\Delta H_{\text{rel}}/R/T} d\Delta H_{\text{rel}} \quad (16)$$

where $g(\Delta H_{\text{rel}})$ gives the relative contribution of the barrier height ΔH_{rel} to k_2 . The simplest assumption is a Gaussian distribution of $g(\Delta H_{\text{rel}})$. In order to fit the experimental data, one has then to determine the parameters ΔH_0 and σ , which are the center and the width of the Gaussian distribution, respectively, and ΔS_{rel} . Another alternative would be a Γ distribution [45]. However, for our interpretation of the data, we used another possibility. The time course of unrelaxed molecules $n(t)$ was fitted with the stretched exponential:

$$n(t) = n_0 \cdot \exp\left(-(p_F(T) \cdot k_2 t)^\beta\right) \quad (17)$$

where k_2 is given by Eq. 15. β and ΔG_{rel} are the fit parameters. This treatment avoids making a guess like a Gaussian for the distribution of the barrier heights. The fit is performed in the time domain. The results are shown in Fig. 7. Afterwards, the energy distribution is obtained by a Laplace transformation. The Laplace transformation from the time to the rate domain was performed according to [46, 47]. Afterwards, a substitution of the rate by the enthalpy gives the following distribution of $g(\Delta H_{\text{rel}})$:

$$g(\Delta H_{\text{rel}}) = -\frac{1}{\pi RT} \cdot \frac{1}{e^{-\frac{\Delta H_{\text{rel}0} - \Delta H_{\text{rel}}}{RT}}} \sum_{m=0}^{\infty} \frac{(-1)^m}{m!} \cdot \sin(\pi\beta m) \cdot \Gamma(\beta m + 1) \cdot \left(e^{-\frac{\Delta H_{\text{rel}0} - \Delta H_{\text{rel}}}{RT}}\right)^{\beta m + 1} \quad (18)$$

where Γ is the gamma function. For the data at 174 and 180 K, we allowed a shift of the barrier-height distribution to higher enthalpies during the experiments. This reflects a structural relaxation within the metastable state.

$$\Delta G_{\text{rel}} = -T\Delta S_{\text{rel}} + \Delta H_{\text{rel}0} + \Delta\Delta H_{\text{rel}} \cdot \left(1 - e^{-(k^* t)^\beta}\right) \quad \text{with} \quad k^* = A^* e^{-(E^*/R/T)^2}. \quad (19)$$

At the beginning of the decay of the metastable state, the enthalpy is $\Delta H_{\text{rel}0}$. During the experiment, the enthalpy increases to the maximum of $\Delta H_{\text{rel}0} + \Delta\Delta H_{\text{rel}}$. The obtained parameters are presented in Table 1. A comparison of theory and experiment is shown in

Fig. 8 Laplace transformation of the stretched exponentials of Fig. 7 (see Eq. 18 and data in Table 1). Solid, dashed, dotted, dashed dotted, and dashed double dotted lines represent the distributions for 147, 174, 180, 190, and 195 K, respectively. For 174 and 180 K, only the starting distribution is given

Fig. 7. A Laplace transformation of the stretched exponential for each temperature gives the enthalpy distributions given in Fig. 8.

8 Discussion

In this contribution, we tried to give a consistent interpretation of experimental results obtained with very different methods. The experimental methods differed in the energy resolution and, hence, in the time resolution. This allows the separation of different contributions to the protein dynamics. As a consequence of the reviewed results, we propose also a new model for an understanding of the conformational transition. Its main aim is to connect conformational changes and protein-specific fluctuations at physiological temperatures. The heme iron is used as a marker in most of the discussed experiments.

The mean square displacements obtained by X-ray structure analysis reveal the well-known conformational substate introduced by Frauenfelder and coworkers [5, 48]. It should be mentioned that especially the $\langle x_{\text{stat}}^2 \rangle^X$ values obtained from the extrapolation to $T = 0$ depend strongly on the resolution of the dataset and on the refinement procedure [49]. Therefore, we have not undertaken a detailed discussion here. The use of synchrotron radiation in combination with the phonon-assisted Mössbauer effect allowed the study of vibrational motions within the conformational substates. The experiments prove that the $\langle x^2 \rangle^Y$ values below T_c measured by Mössbauer spectroscopy are essentially caused by fast motions [17]. The results of the phonon-assisted Mössbauer effect show that the fast motions are also present at room temperature. The $\langle x_{\text{vib}}^2 \rangle^Y$ values increase linearly with temperature up to room temperature. A normal mode analysis explains $\langle x_{\text{vib}}^2 \rangle^Y$ very well. Fast motions can, however, not explain the mean square displacements measured at the iron by Mössbauer absorption spectroscopy above T_c . In this temperature region, slow quasidiffusive motions contribute to the mean square displacements which cannot be seen in X-ray structure analysis and in the phonon-assisted Mössbauer effect due to the poor energy resolution of these methods. RSMR experiments suggest that the quasidiffusive motions measured at the iron are characteristic for the whole protein [38, 50]. The diffusion involves a collective motion of segments of the size of α -helices. It is limited in space and the fluctuations occur around the average structure obtained by X-ray structure analysis.

In our picture, there exists a temperature-dependent equilibrium between the concentrations f and cs of molecules in the flexible state F and in the conformational substates CS, respectively (compare Eq. 12). The temperature dependence of the population of the flexible state is determined from the Mössbauer absorption experiments. Starting with an average energy $3/2RT$ of a conformational substate, an average ΔG_{flex} with the contribution $\Delta H_{\text{flex}} = 34.9$ kJ/mol and $\Delta S_{\text{flex}} = 0.121$ kJ/mol/K is necessary to reach the flexible state. Note the large positive entropy. In comparison to the ground state, the flexible state has many additional *degrees of freedom*, which is essential for its population. If a molecule is already in the flexible basin, it is obvious to assume that it can start the conformational transition from this state. If a molecule is in a stable conformation (e.g., MbCO or Mbmet), it reaches the flexible state with the described probability, performs Brownian oscillations, and is trapped again in a CS. If it is in a metastable conformation (e.g., Mb* + CO which means CO is photolyzed, but Mb* has still essentially the structure of MbCO, or Mb(II,ls,w)), a molecule in the flexible state has two possibilities: either it is trapped again in a CS or it surmounts a barrier ΔG_{rel} , changes conformation and ends in a new stable conformation (e.g., Mbdeoxy). Since the structural fluctuations in the flexible state can be described by

overdamped Brownian oscillations, it is obvious that Kramers' theory has to be applied for the conformational transition.

At 174 and 180 K, there is an indication for a further relaxation process in addition to the conformational transition into *R*. Following the treatment of [51], we assumed that this is a structural relaxation within the metastable state Mb(II,ls,w). A similar effect was also seen in [39, 52]. The time dependence of k^* was also measured by [51, 53, 54] for the rebinding of CO to myoglobin. These authors obtain $A^* = 10^{18} \text{ s}^{-1}$, $E^* = 10000 \text{ J mol}^{-1}$, and $\beta^* = 0.24$. However, our data for H₂O dissociation were best fitted with $A^* = 10 \text{ h}^{-1}$, $E^* = 3,500 \text{ J mol}^{-1}$, and $\beta^* = 1$. In Sample 2, the distribution width is clearly decreased at 195 K. This is a clear indication of motional narrowing. The increase of ΔS_{rel} from state *F* to the top of the barrier between *F* and *R* may have two reasons. First of all, in the transition state, the water is no longer bound to the iron, gaining, therefore, degrees of motional freedom. Another source of the increase in entropy may come from the fact that the molecules have an increased flexibility at the transition state.

In the literature, there exist simpler theories to describe conformational transitions [28, 55]. We refer to our own treatment published in [41]. Why do we need another theory? One and the same experimental dataset can often be explained by different physical pictures. Each successful description offers only a possibility but no proof of correctness. Without comparing with other theories in any detail, the introduced picture is the only one which connects structural fluctuations within one conformation with conformational transitions into another conformation. It is a necessary consequence of the existence of a flexible state.

Since some years, the conformational relaxation can be investigated by time-resolved X-ray structure determination using synchrotron radiation. Such an experiment has been performed on the myoglobin mutant L29W [56]. Essential relaxations occur for times less than 1 ns up to microseconds. The physical picture described in this contribution may be helpful to disentangle the different effects resulting in these conformational relaxations.

It should be emphasized that our treatment of the conformational transition is only preliminary. Due to the limitation of available experimental data, we were forced to combine results obtained on Mb(II,ls,w), Mbmet, and MbCO. Moreover, physical arguments had to be used to limit the parameter field and to achieve consistency among all experiments available.

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