Eur Biophys J (1985) 12:167–172

European Biophysics Journal

Determination of the second order doppler shift of iron in myoglobin by Mössbauer spectroscopy

L. Reinisch, J. Heidemeier, and F. Parak*

Institut für Physikalische Chemie, Universität Münster, Schloßplatz 4–7, D-4400 Münster, Federal Republic of Germany

Received October 29, 1984/Accepted February 26, 1985

Abstract. We have performed Mössbauer absorption experiments on a sample of deoxygenated myoglobin crystals from 5 K to 280 K. With two series of measurements, one with the source and sample at the same temperature and the other with the source always at 298 K, we are able to extract information from the second-order Doppler effect in the sample. Simple models consistent with a description of myoglobin with low lying electronic states which are thermally populated above 40 K indicate that the Debye temperature of myoglobin is 220 K, in agreement with measurements using the Lamb-Mössbauer factor. The second-order Doppler effect is proportional to the square of the velocity of the motion. We are unable to see any indication of protein specific motion from the second-order Doppler effect, thereby indicating that protein specific motions are relatively slow.

Key words: Protein dynamics, Mössbauer spectroscopy

Introduction

Fluctuations in protein molecules, particularly myoglobin, have been shown to be significant for their function (Austin et al. 1975). Various aspects of the protein fluctuations have been studied. The rates for ligand recombination with myoglobin are slowed by viscous solvents (Beece et al. 1980). The experiments give evidence for a definite damping of the necessary protein fluctuations by the solvent. In addition, Mössbauer absorption spectroscopy measurements have analyzed the Lamb-Mössbauer factor and the line width to give detailed information about protein fluctuations at the iron atom in myoglobin (Parak et al. 1982; Knapp et al. 1982; Bauminger et al. 1983). Mössbauer effect measurements have given both amplitude and a time threshold for the motions. The model developed from the Mössbauer measurements, of a restricted overdamped Brownian oscillator (Parak and Knapp 1984), represents a picture of myoglobin consistent with the experimental data.

Most conclusions from Mössbauer experiments are based on the determination of the Lamb-Mössbauer factor,

$$f = \exp\left(-\langle x^2\rangle k^2\right), \tag{1}$$

which gives the probability for recoil free absorption. Here, k is $2\pi/\lambda$ with $\lambda = 0.86$ Å for the 14.4 keV transition in ^{57}Fe, and $\langle x^2\rangle$ is the mean-squared displacement of the absorbing atom. The Lamb-Mössbauer factor is equal to one if the absorbing atom sits completely motionless, $\langle x^2\rangle = 0$. Larger amplitude motions of the absorbing atom result in less Mössbauer absorption. The Mössbauer photons from a ^{57}Co source have a 10^{-7} s lifetime, so that the $\langle x^2\rangle$ value applies only to motions considerably faster than the 10^{-7} s lifetime. Motions that occur with a period near 10^{-7} s pose a special problem. They do not influence the Lamb-Mössbauer factor, but they do, however, cause a broadening of the typical narrow Mössbauer absorption lines.

The information from Mössbauer spectroscopy is not necessarily limited to the determination of the Lamb-Mössbauer factor and the spectral distribution of absorption lines. The motion of the iron atom in myoglobin will cause an energy shift from the Doppler effect, similar to the energy shift of the source mounted on a Mössbauer drive unit. Isotropic linear motions in the sample will clearly average to zero; nevertheless, a second-order Doppler effect remains. Here the energy shift is proportional

* To whom offprint requests should be addressed

Abbreviations: Mb, Myoglobin

to the square of the velocity of the atoms (Pound and Rebka 1960; Josephson 1960). The second-order Doppler shift can be used as another parameter in the description of the dynamic properties of iron and its environment.

To better understand the motion of the atoms in proteins, this paper reports investigations of the second-order Doppler effect in a sample of ^{57}Fe enriched deoxygenated myoglobin crystals. The velocity and the motion of the heme iron atom are influenced by the protein motion. However, exact separation of a temperature-dependent isomer shift and the second-order Doppler effect is nearly impossible. Therefore, the temperature dependence of both the deoxygenated myoglobin sample and the ^{57}CoRh source has to be measured and analyzed. Using simple models, limits can be put on the information from the second-order Doppler effect.

Experimental

Sperm whale myoglobin was enriched in ^{57}Fe according to Parak and Formanek (1971) and crystallized. The metmyoglobin crystals were reduced by sodium dithionite under anaerobic conditions. The crystals were sealed in a plastic sample holder with no excess mother liquid.

Conventional gamma resonance spectrometers with a sinusoidal velocity profile were used. In one system the sample and the source temperature were varied so that both always had the same temperature. In the other system, the source was always at room temperature, and the sample temperature was varied. Both systems used a cryostat for cooling with temperature stabilized to better than $\pm$ 2 K. The experiments were all performed with the same ^{57}CoRh source and with a proportional counter. The 14.4 keV radiation was separated with a single channel analyzer.

Earlier studies of deoxygenated myoglobin crystals (Parak et al. 1982) have shown the need for four absorption lines to fully describe the absorption spectrum. Centreed about each of the two narrow lines is a very wide absorption line. These wide lines are only apparent in spectra with maximum velocities near $\pm$ 30 mm/s. To maintain the needed energy resolution, measurements were made with maximum velocites near $\pm$ 3 mm/s. Thereby, the wide absorption lines are intentionally ignored. Because the major concern, here, is the energy shift of the lines, ignoring the wide absorption lines does not influence the results.

The measured Mössbauer absorption spectrum of deoxygenated myoglobin is a quadrupole doublet. The single absorption energy is split into two lines, each with a similar absorption probability. The energy shift is then defined by the symmetric centre of the two lines. In addition, a small fraction of the sample had partially denatured. The distal histidine forms a bond with the heme iron atom. The denatured myoglobin was detected as an additional quadrupole doublet, this doublet was also fitted to each spectrum but not used in the analysis. The fraction of the sample present as denatured protein was nearly constant at low temperatures (below about 240 K). However, at higher temperatures (where the mother liquid melts), the fraction of denatured myoglobin increased with time.

Results and discussion

The upper part of Fig. 1 shows the shift of the Mössbauer absorption lines of the sample with respect to the ^{57}CoRh source as a function of temperature, T. As usual, this shift is the energy which must be added to the source in order to match the absorption energy of the sample. This energy shift is reported as the Doppler velocity, v, and can be converted into conventional energy units (e.g. keV) by dividing by the speed of light, c, and multiplying by the energy of the gamma photons ($E_\gamma = 14.4$ keV). In order to avoid confusion, all equations which refer to convential energy units are marked with a prime ($'$), whereas equations referring to energy shifts in units of Doppler velocity are left without a prime.

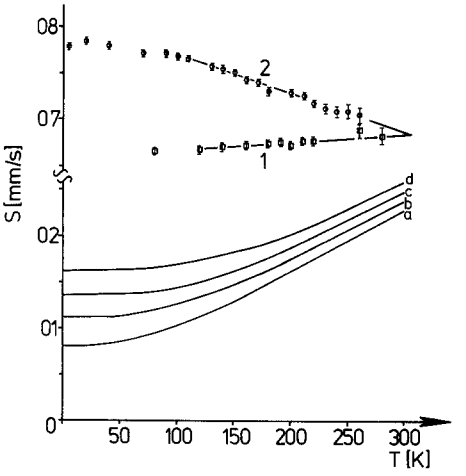

Fig. 1. The measured Mössbauer shifts as a function of sample temperature T from a sample of deoxygenated myoglobin crystals with respect to a ^{57}CoRh source. Open circles ($S_2(T)$) are from measurements with the source at 298 K. The squares ($S_1(T)$) are from measurements with the source and the sample at the same temperature. The lines are least-squares fits of a linear temperature dependence to the data from 100 K to 300 K. In the lower part the calculated second-order Doppler shift of the source for different Debye-temperatures is shown. a) $\Theta_D = 300$ K, b) $\Theta_D = 415$ K, c) $\Theta_D = 500$ K, d) $\Theta_D = 600$ K

The data presented is for two series of measurements. The first measurements were made with the sample and the source at the same temperature; the second series of measurements were made with the source at 298 K and varying the sample temperature. As is obvious from Fig. 1, there is no pronounced change in the temperature dependence of the energy shift at temperatures higher than approximately 200 K. Therefore, there is an absence of any direct indication of protein specific motions influencing the energy shifts.

The mismatch in energy between the source and the sample is due to two contributions. The iron in the source and in the absorber is in different chemical environments, which causes different electron densities near the nucleus, giving rise to the well known isomer shift, S_{iso}. If the electronic level scheme of the iron contains energetically low lying levels which can be thermally populated in the temperature range under investigation, the isomer shift becomes temperature-dependent. In both the source and the absorber the iron is not at rest but performs vibrations. Assuming that the displacements occur periodically with a mean squared velocity $\langle u^2 \rangle$, one obtains the second-order Doppler shift

$$S'_{\mathrm{soD}} = -E_\gamma \frac{\langle u^2 \rangle}{2c^2}. \tag{2}$$

Since $\langle u^2 \rangle$ is proportional to the mean kinetic energy of the iron and the mean kinetic energy increases with temperature, the second-order Doppler shift depends on temperature.

The energy shift in a Mössbauer spectrum for the case of sample and source at the same temperature can then be written as

$$S_1(T) = S_{\mathrm{iso}}^{\mathrm{Rh}}(T) + S_{\mathrm{soD}}^{\mathrm{Rh}}(T) + S_{\mathrm{iso}}^{\mathrm{Mb}}(T) + S_{\mathrm{soD}}^{\mathrm{Mb}}(T). \tag{3}$$

Here, $S_1(T)$ is the observed energy shift. $S_{\mathrm{iso}}^{\mathrm{Rh}}(T) + S_{\mathrm{iso}}^{\mathrm{Mb}}(T)$ gives the isomer shift between the ^{57}CoRh source and the Mb absorber. Since the energy difference between the ground state and the excited state of the Fe nucleus in Rh is smaller than the energy difference of the Fe nucleus in Mb, the isomer shift is a positive Doppler velocity. $S_{\mathrm{soD}}(T)$ refers to the second-order Doppler shift with respect to a hypothetical environment of rigidly fixed iron atoms. According to Eq. (2), a mean squared velocity, $\langle u^2 \rangle$, effectively reduces the energy difference between the excited and the ground state of the ^{57}Fe nucleus. In the source this energy loss of the emitted gamma photon has to be compensated with a positive Doppler velocity, v, of the Mössbauer spectrometer, yielding a positive $S_{\mathrm{soD}}^{\mathrm{Rh}}(T)$. In the absorber, $S_{\mathrm{soD}}^{\mathrm{Mb}}(T)$ corresponds to a negative Doppler velocity, v. The subscript 1 correlates this equation with line 1 in Fig. 1.

The energy shift in the Mössbauer spectrum for the case of source at constant temperature ($T_0 = 298$ K) can be written as

$$S_2(T) = S_{\mathrm{iso}}^{\mathrm{Rh}}(T_0) + S_{\mathrm{soD}}^{\mathrm{Rh}}(T_0) + S_{\mathrm{iso}}^{\mathrm{Mb}}(T) + S_{\mathrm{soD}}^{\mathrm{Mb}}(T). \tag{4}$$

Line 2 is also shown in Fig. 1.

Source

First, the source is considered. Since the ^{57}Fe is in a metal rhodium matrix, the chemical environment, or the isomer shift, is determined by the electron structure of the rhodium. Below 300 K, thermal energy is normally too small to significantly populate higher electronic states in a metal. In this temperature range the electron structure is temperature-independent, and the isomer shift should also be temperature-independent. Therefore, in the first attempt, the source is described with a constant isomer shift. Differentiating Eqs. (3) and (4) with respect to temperature and taking the difference, one obtains

$$\frac{\mathrm{d}}{\mathrm{d}T} S_2(T) - \frac{\mathrm{d}}{\mathrm{d}T} S_1(T) = \frac{\mathrm{d}}{\mathrm{d}T} S_{\mathrm{soD}}^{\mathrm{Rh}}(T) \tag{5}$$

or the slope of line 2 minus the slope of line 1 is the change in the second-order Doppler effect with temperature. In a Debye model the mean square velocity is given by

$$\langle u^2 \rangle = \frac{9R}{8 M_{\mathrm{Fe}} c^2} \left[\Theta_D + 8T \left(\frac{T}{\Theta_D} \right)^3 \int_0^{\Theta_D/T} \frac{x^3 \, \mathrm{d}x}{e^x - 1} \right]. \tag{6}$$

The temperature dependence of the second-order Doppler effect is then

$$\frac{\mathrm{d}}{\mathrm{d}T} S'_{\mathrm{soD}} = \frac{-9 R E_\gamma}{2 M_{\mathrm{Fe}} c^2} \left(\frac{T}{\Theta_D} \right)^3 \int_0^{\Theta_D/T} \frac{x^4 e^x \, \mathrm{d}x}{(e^x - 1)^2}, \tag{7}$$

where R is the molar gas constant (8.31 J mole^{-1} K^{-1}), M_{Fe} is the atomic mass of ^{57}Fe (0.057 kg/mole) and c is the speed of light. Θ_D is the Debye temperature of the source. Fitting Eqs. (5) and (7) to the data, $\Theta_D = 415 \pm 15$ K is found. The Debye temperature of rhodium has been reported as 427 K (Steyert and Taylor 1964). It has to be noted that their determination used the Lamb-Mössbauer factor and therefore the temperature dependence of the mean squared displacement, $\langle x^2 \rangle$; whereas our determination came from the second-order Doppler shift via the temperature dependence of the mean squared velocity, $\langle u^2 \rangle$. The agreement of our results with the literature value is good, it is therefore felt that the description of the source with a temperature-independent isomer shift is valid.

From Θ_D, the absolute energy shift, $S_{\mathrm{soD}}^{\mathrm{Rh}}$, from the second-order Doppler effect can be found from

Eqs. (2) and (6). In the lower part Fig. 1, the shift from the second-order Doppler effect for $\Theta_D = 300$, 415, 500 and 600 K are shown. Using this figure, measured energy shifts can be corrected for the energy shift from the second-order Doppler effect in a rhodium source.

Deoxygenated myoglobin

Equation (4) is differentiated with respect to the temperature. First a model similar to that used to describe the source is employed, and $\frac{d}{dT} S_{iso}^{Mb} = 0$ for T below 300 K. Then using Eq. (7), the Debye temperature can be found, such a fit yields a Debye temperature of 530 ± 25 K. This value would indicate that myoglobin has a higher Debye temperature than rhodium, which in turn suggests that myoglobin is more rigid than rhodium. Such a result is very unreasonable for a protein and therefore, the assumption that $\frac{d}{dT} S_{iso}^{Mb} = 0$ must be abandoned. For myoglobin the temperature dependence of the isomer shift cannot be neglected. The electronic structure of the iron atom has energetically low lying levels close to the ground state. These low lying levels are thermally populated at temperatures well below room temperature (Eicher et al. 1974). Since the electronic relaxation between the energy levels is fast in comparison to the nuclear life time, the Mössbauer absorption reflects an average electron density which is the superposition of the contributions from the different levels weighted with a Boltzmann population.

Trautwein et al. (1970) have calculated the isomer shift of deoxygenated myoglobin from the energy-level scheme of the iron nucleus. The assumptions used in these calculations are, however, rather crude. In the case of ^{57}Fe, no sufficiently accurate theory exists to enable calculation of absolute values of the isomer shift from the electronic level scheme. Therefore, a more phenomenological description of the temperature dependence of the isomer shift is used here. Approximately, one may allow the isomer shift from myoglobin to have a linear temperature dependence. To keep the model physically meaningful, the isomer shift needs to be temperature-independent below a certain temperature, T_c. At this temperature, higher electronic states are no longer thermally populated. Therefore, there are now three parameters: the Debye temperature, Θ_D; the critical temperature for the isomer shift, T_c; and the slope of the linear temperature dependence of the isomer shift. The Debye temperature, Θ_D, is strongly dependent on T_c. In the approximation of a temperature-independent isomer shift used first, T_c was essentially equal to 280 K. Taking the other extreme case of $T_c = 0$ K, $\Theta_D = 170 \pm 15$ K is found.

Fitting the data to the three parameters in the model does not necessarily give the best description of the system. The range of values which fit the data within the error limits is large. In Fig. 2 the Debye temperature is plotted as a function of the the critical temperature, T_c. With T_c above 200 K, the fits are only slightly worse than with T_c below 200 K. The χ^2 of the fits with T_c below 200 K vary by only 50% with a minimum near $T_c = 60$ K. The population of the electronic states is also reflected in the quadrupole splitting. In deoxygenated myoglobin, the quadrupole splitting is almost tempera-

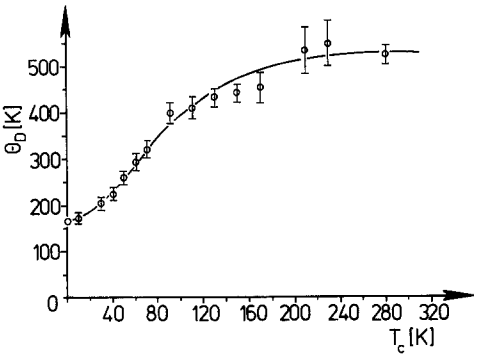

Fig. 2. Calculated Debye temperatures (Θ_D) as a function of the critical temperature, T_c (as explained in the text). The error bars represent ranges of Debye temperatures that fit the data. The solid line is drawn to guide the eye

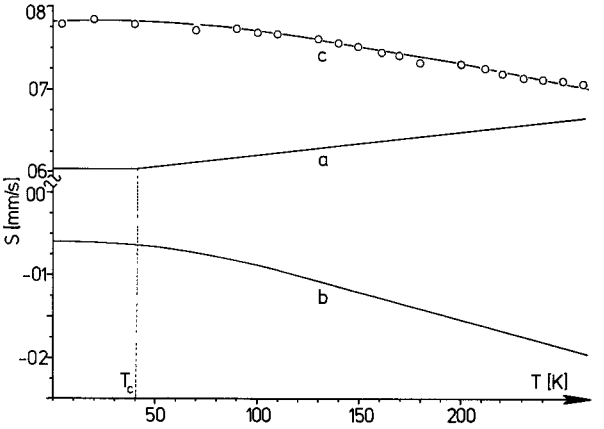

Fig. 3. Interpretation of the temperature dependence of the total Mössbauer shift of deoxygenated myoglobin crystals with the source at 298 K. Curve a gives the calculated isomer shift as a function of temperature assuming $T_c = 40$ K. Curve b gives the second-order Doppler shift of myoglobin as a function of temperature with $\Theta_D = 220$ K. Together with a constant second-order Doppler shift of the source (+ = 0.236 mm/s) curve a plus b yield the solid line c which fits the total measured Mössbauer shift of the myoglobin crystals. Line c represents a least squares fit to the experimental data, where T_c was fixed and Θ_D and the slope of the isomer shift were varied

ture-independent below 40 K (Eicher et al. 1974), indicating little or no thermal population of higher electronic states at temperatures below 40 K. T_c is therefore taken to be equal to 40 K.

If 40 K for T_c is used, $\Theta_D = 220 \pm 15$ K is found. The fit from this model is shown in Fig. 3. The Debye temperature from this fit also correlates well with the range of Debye temperatures from Dwivedi et al. (1979). It should again be emphasized that in this work, the Debye temperature was determined from the Lamb-Mössbauer factor below 200 K.

At 298 K for both Mb and Rh, one is close to the high temperature region of the second-order Doppler shift where, in the Debye approximation, $\langle u^2 \rangle$ increases linearly with temperature and is independent of Θ_D. Therefore, $S_{soD}^{Rh}(298\,K) + S_{soD}^{Mb}(298\,K)$[1] becomes negligible and according to Eq. (4) the true isomer shift between the ^{57}CoRh source and the Mb absorber is measured. The measured isomer shift is then +0.68 mm/s (+0.794 mm/s after the usual normalization with respect to an Fe-metal source is made).

Protein specific motion

The mean squared displacement of the iron atom in deoxygenated myoglobin, determined by the Mössbauer effect, increases dramatically for temperatures above 200 K (Parak et al. 1981; Parak et al. 1982; Bauminger et al. 1983). The increase is attributed to protein specific motions, which are thermally prevented ("frozen-out") at lower temperatures. The mean squared displacement of the iron atom was determined from the Lamb-Mössbauer factor. Although the second-order Doppler shift is totally dependent upon atomic motion, no change in the temperature dependence near 200 K is noted. Why is it not present? The Lamb-Mössbauer factor as shown in Eq. (1) is dependent upon the amplitude of the atomic motion. The second-order Doppler effect is dependent upon the square of the velocity of the motion. Although the protein specific motions have a very large amplitude, the velocities of the motions are quite small.

The velocities of atomic oscillations and protein specific motions can be compared. The time for atomic oscillations can be taken from the observed Raman lines for deoxygenated hemoglobin. Fe-N_ε has a Raman line associated with vibrational motion at 364 cm^{-1} (Brunner and Sussner 1973). Considering an amplitude for atomic vibrations of about 0.145 Å ($\langle x_v^2 \rangle = 0.021$ Å^2), a velocity squared of 2.5×10^4 m^2/s^2 can be calculated. The determination of the second-order Doppler shift of about 0.2 mm/s at 280 K (within 10%, this value is independent of T_c) corresponds to a squared velocity for the iron of 1.2×10^5 m^2/s^2. The factor of 5 difference between the velocity squared determined from the second-order Doppler shift and the simplistic calculation is amazingly small. The estimation verifies once more that $\langle x_v^2 \rangle$ as determined by Parak et al. (1982) arises from normal solid state vibrations. On the other hand, the $\langle x_l^2 \rangle$ measured at the iron atom in myoglobin due to protein specific Brownian motion is about 0.034 Å^2. The time for the motion determined from Mössbauer effect measurements is near 10^{-8} s. The velocity squared from the protein specific motions is 3.4×10^{-6} m^2/s^2. Since the second-order Doppler shift is proportional to the velocity squared, it is obvious that the protein specific contribution, in contrast to the atomic vibrational contribution, is not measureable. It is appropriate to note here the importance of the time factors for protein motion. If all modes of protein motions were as fast as computer simulations predicted, starting at 150 cm^{-1} (Levy et al. 1982), then protein specific motions would have velocities comparable to atomic vibrations, and the second-order Doppler effect should be noticeably influenced. The measurements, here, support the interpretation of the broad Mössbauer absorption lines representing slow protein specific motion which are generally much slower than atomic vibrations.

Conclusions

The determination of the Debye temperature of deoxygenated myoglobin crystals using the second-order Doppler effect agrees well with measurements using the Lamb-Mössbauer factor below 200 K. A temperature-dependent isomer shift for temperatures above about 40 K has to be assumed. This is in agreement with the findings of low energy levels for the heme iron which are thermally populated. The absence of any protein specific second-order Doppler effect is not surprising when considering the slow nature of the motion as previously determined.

The present results strengthen these investigations. They show once more, that at the present time computer simulated protein dynamics omit important parts of the protein specific motions.

Acknowledgements. This investigation was stimulated by discussions with P. Debrunner. One of us (L.R.) thanks the Alexander von Humboldt Foundation for a stipendium during the time of this research. The work was supported by the Deutsche Forschungsgemeinschaft.

1 Note that the sign of S_{soD}^{Rh} for the source and S_{soD}^{Mb} for the absorber is different

References

Austin RH, Beeson KW, Eisenstein L, Frauenfelder H, Gunsalus IC (1975) Dynamics of ligand binding to myoglobin. Biochemistry 14:5355–5373

Bauminger ER, Cohen SG, Nowik I, Ofer S, Yariv J (1983) Dynamics of heme iron in crystals of metmyoglobin and deoxymyoglobin. Proc Natl Acad Sci USA 80:736–740

Beece D, Eisenstein L, Frauenfelder H, Good D, Marden MC, Reinisch L, Reynolds AH, Sorensen LB, Yue KT (1980) Solvent viscosity and protein dynamics. Biochemistry 19: 5147–5157

Brunner H, Sussner H (1973) Resonance Raman scattering on haemoglobin. Biochim Biophys Acta 310:20–31

Dwivedi A, Pederson T, Debrunner P (1979) Recoilless fraction of iron proteins in frozen solution. J Phys (Paris) C2: 531–533

Eicher H, Bade D, Parak F (1974) Theoretical determination of the electronic structure and the spatial arrangement of ferrous iron in deoxygenated sperm whale myoglobin and human haemoglobin from Mössbauer experiments. J Chem Phys 64:1446–1455

Josephson BD (1960) Temperature-dependent shift of γ-rays emitted by a solid. Phys Rev Lett 4:341–342

Knapp EW, Fischer SF, Parak F (1982) Protein dynamics from Mössbauer spectra. The temperature dependence. J Chem Phys 86:5042–5047

Levy RM, Perahia D, Karplus M (1982) Molecular dynamics of an α-helical polypeptide: Temperature dependence and deviation from harmonic behavior. Proc Natl Acad Sci USA 79:1346–1350

Parak F, Formanek H (1971) Untersuchung des Schwingungsanteils und des Kristallgitterfehleranteils des Temperaturfaktors in Myoglobin durch Vergleich von Mössbauerabsorptionsmessungen mit Röntgenstrukturdaten. Acta Crystallogr Sect A 27:573–578

Parak F, Frolov EN, Mössbauer RL, Goldanskii VI (1981) Dynamics of metmyoglobin crystals investigated by nuclear gamma resonance absorption. J Mol Biol 145:825–833

Parak F, Knapp EW, Kucheida D (1982) Protein dynamics Mössbauer spectroscopy on deoxymyoglobin crystals. J Mol Biol 161:177–194

Parak F, Knapp EW (1984) A consistant picture of protein dynamics. Proc Natl Acad Sci USA 81:7088–7092

Pound RV, Rebka GA (1960) Variation of the energy recoil-free gamma rays from solids. Phys Rev Lett 4:274–275

Steyert WA, Taylor RD (1964) Lattice dynamical studies using absolute measurements of the Lamb-Mössbauer recoil-free fraction. Phys Rev 134:A716–722

Trautwein A, Eicher H, Mayer A (1970) Electronic structure, quadrupole splitting, chemical shift, and susceptibility of ferrous iron in anhydrohemoglobin, anhydromyoglobin, and bispyridinehemin. J Chem Phys 52:2473–2477