Eur Biophys J (1996) 25: 43–46

BIOPHYSICS LETTER

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Temperature dependent inelastic X-ray scattering of synchrotron radiation on myoglobin analyzed by the Mössbauer effect

Received: 17 May 1996 / Accepted: 11 June 1996

A new method of inelastic X-ray scattering of synchrotron radiation with an energy analysis by the Mössbauer effect was used to measure protein dynamics. This method is comparable to incoherent neutron scattering but gives information on all atoms of the sample. Myoglobin was hydrated to 0.4 g H_2O/g protein and measured at temperatures of 62, 124, 189, and 300 K. The shapes of the energy spectra were approximated within an Einstein model using results of a normal mode analysis of myoglobin. This simple model described the spectra quite well using only one average slope of the mean square displacement with temperature. It shows, together with results of Mössbauer absorption experiments, that protein dynamics comes from a superposition of two types of motion. Harmonic vibrations are present in the whole temperature range, protein specific modes above 200 K give rise to quasielastic scattering.

Protein molecules have a well defined but nevertheless flexible structure. Both features are essential for their functions. The dynamic properties of proteins are best investigated in myoglobin. For a review we refer to Frauenfelder et al. (1988) or Parak and Frauenfelder (1993). Here, we want to describe a new method for the investigation of protein dynamics using inelastic X-ray scattering of synchrotron radiation as described by Chumakov et al. (1996). In the case of proteins this method can be compared with incoherent neutron scattering (Cusack and Doster 1990). In contrast to the incoherent scattering of neutrons, which averages over the motions of the H-atoms, this new method is sensitive to all atoms of the sample. The method is based on the following principle: X-rays from a synchrotron fall on the sample under investigation and are scattered elastically and inelastically. The energy of the incident radiation is varied in the vicinity of the nuclear transition of ^{57}Fe (14.4 keV). The energy of the radiation scattered by the sample is analyzed by the Mössbauer effect in a ^{57}Fe foil.

In an inelastic scattering process an X-ray quantum gains or loses energy by interactions with the modes of motion in the sample. If this energy change is just the right one to reach the Mössbauer resonance energy of ^{57}Fe, the radiation scattered by the sample can also be resonantly forward scattered in an analyzer containing ^{57}Fe nuclei. This nuclear scattering leads to a time delayed radiation, the intensity of which is proportional to the density of motional modes in the sample with the proper energy.

The experiment was performed at the nuclear resonance beamline ID18 (Rüffer and Chumakov 1996) at the European Synchrotron Radiation Facility in Grenoble. The storage ring was run in the 16 bunch mode with a mean current of about 60 mA, yielding X-ray flashes of a length of 65 ps every 176 ns. The experimental setup is shown in Fig. 1. The undulator U23 of 22.8 mm period is optimized for the 14.4 keV energy of the ^{57}Fe Mössbauer level. A silicon double crystal monochromator (M1) reduces the bandwidth to 2.8 eV and a following high resolution 'nested' channelcut monochromator (M2) reduces it further to 4.4 meV. This is so far the highest resolution for a nested monochromator, it is achieved by combination of two Si(422) and two Si(975) reflections. The energy of the radiation was varied in the range ±70 meV with steps of 0.8 meV. The flux of incident radiation on the sample was about $2 \cdot 10^8$ photon/s.

Freeze dried horse heart myoglobin hydrated to 0.4 g water/g protein (corresponding to 400 water molecules per protein molecule) was investigated (S in Fig. 1). It was

The work was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie under contract 05 643 WOC and the DFG (Graduate College FU Berlin).

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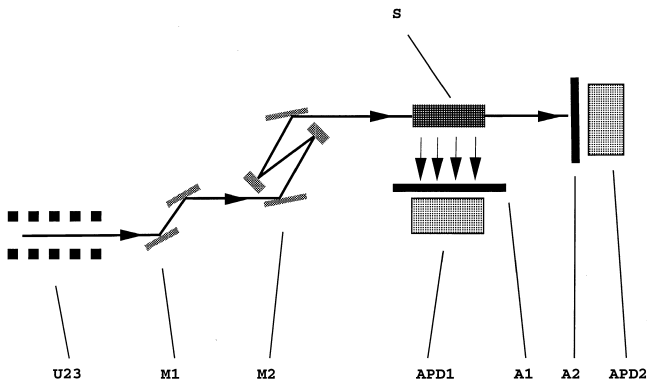

Fig. 1 Setup for the experiment. *U23* – 22.8 mm period undulator, *M1* – Si(111) double crystal monochromator, *M2* – Si(422) and Si(975) high resolution monochromator, APD1/2 – avalanche photodiodes, *A*1/2 – ^{57}Fe metal Mössbauer analyzer foils, *S* – sample

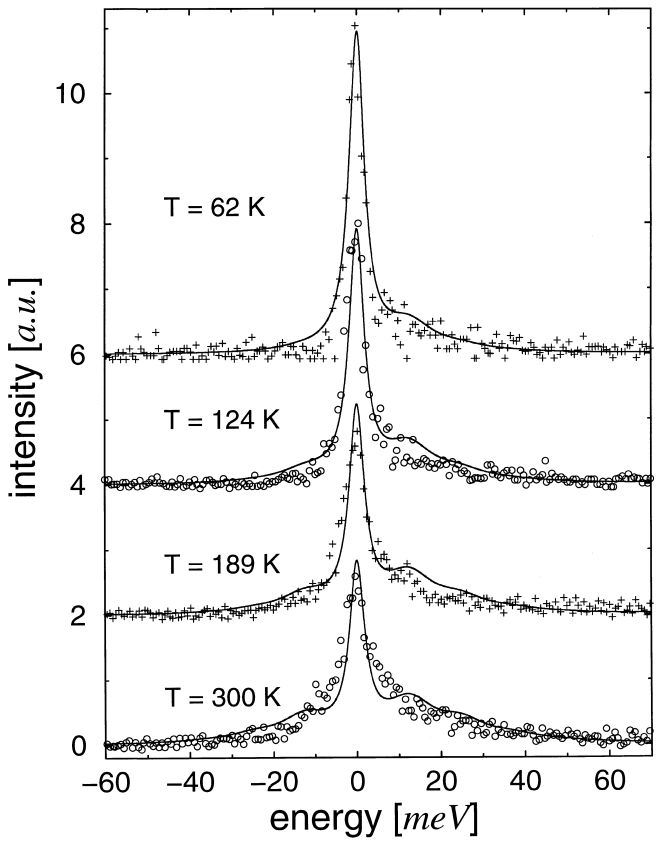

Fig. 2 Energy spectra of inelastic X-ray scattering by hydrated myoglobin at four temperatures. The *solid lines* are calculated spectra using an Einstein frequency $\hbar\omega_1 = 12.4$ meV and a mean square fluctuation $\langle R_1^2 \rangle$ obtained from a normal mode analysis of myoglobin as explained in the text

contained in a sample holder with vacuum tight mylar windows and mounted in a closed cycle refrigerator. The thickness of the sample in the direction of the incoming beam was 10 mm. Slits confined the beam to 1 mm in the horizontal and 0.3 mm in the vertical direction.

Time resolving avalanche photodiodes (APD) (Baron 1995) were used as detectors in the forward and the scattering direction. Iron foils enriched in ^{57}Fe were placed in front of each detector as nuclear resonance energy analyzers. The fraction of radiation exciting the ^{57}Fe nuclear Mössbauer level in the analyzer foil is coherently reemitted in the forward direction with a time delay in the ns range (van Bürck et al. 1992). The rest passes the analyzer foil without delay. The delayed radiation was integrated between 9 and 160 ns after the prompt pulse. The ADP1 detecting the scattered quanta (see Fig. 1) had an effective area of $10 \cdot 10$ mm^2, the distance from the sample was 3.5 mm. This geometry determines the angular range of the detection of the scattered radiation which is not resolved. ADP2 is situated in the forward direction. This detector gives the reference energy and the energy resolution of the method. The hyperfine splitting of the energy levels of the iron metal Mössbauer analyzer is about 0.5 μeV and can, therefore, be neglected compared to the bandwidth of the monochromator.

The energy dependence of inelastic X-ray scattering of myoglobin using nuclear resonance energy analysis is shown in Fig. 2 for four temperatures. The counting rates were about 1 s^{-1} in the maximum of the central peaks. A constant background of 0.018 s^{-1} was measured with closed beam shutters. In Fig. 2 this background has already been subtracted. The experimental intensities given for the lowest three temperatures are normalized to equal incoming intensity and measuring times. Owing to a readjustment of the scattering geometry, for the room temperature intensities the common normalization factor of the calculated spectra had to be enlarged by 11%.

For a first analysis of the data we used a simple model based on an incoherent scattering function which reads in the Gaussian approximation:

$$I_{inc}(k,t) = e^{i\omega_0 t} e^{-(\gamma_0/2)t} \sum_j f_j^2(k)\, e^{-k^2\langle R_j^2\rangle/3} e^{k^2\langle \vec{R}_j(t)\vec{R}_j(0)\rangle/3} \quad (1)$$

Here, ω_0 denotes the frequency of the incident radiation, γ_0 stands for the resolution of the nested monochromator. $\vec{k}$ is the scattering vector determined by $\vec{k} = \vec{k}_1 - \vec{k}_0$ where $\vec{k}_0$ and $\vec{k}_1$ are the wavevectors for the incoming and outgoing radiation respectively with $|\vec{k}_0| = |\vec{k}_1| = 7.3$ Å^{-1}. f_j is the atomic form factor and $\vec{R}_j$ the coordinate of atom j. For an explanation we refer, for instance, to Springer (1972) or Mössbauer (1987). Assuming Lorentzians for the vibrational modes in the frequency domain the correlation function in the time domain reads

$$\langle \vec{R}_j(t)\vec{R}_j(0)\rangle = \sum_m \frac{\langle R_j^{m^2}\rangle}{1+a_m} (\exp(t(i\omega_m - \gamma_1/2)) + a_m \exp(t(-i\omega_m - \gamma_1/2))) \quad (2)$$

where the Boltzmann factor of the normal mode m is $a_m = \exp(-\hbar\omega_m/(k_B T))$ and $\langle R_j^{m2}\rangle$ is the amplitude squared contribution of normal mode m at atom j. The first expo-

nential term in Eq. (2) describes excitation, the second de-excitation of a phonon of frequency ω_m. The linewidth parameter γ_l stands for the limited lifetime of the phonon modes.

In a first approximation one may use the Einstein model with a single vibrational mode and one effective atom type, j = 1, m = 1. Then, Fourier transformation from the time to the frequency domain yields

$$S_{inc}(k,\omega) = f_1^2(k)\, e^{-k^2\langle R_1^2\rangle/3} \sum_{N=0}^{\infty} \frac{1}{N!}\left(\frac{k^2\langle R_1^2\rangle}{3(1+a_1)}\right)^N J_1^{(N)}(\omega) \quad (3)$$

with $J_1^{(N)}(\omega) = \sum_{n=0}^{N} \binom{N}{n} a_1^n L_N[\omega]$. The term $L_N[\omega] = \frac{1}{\pi} \frac{(\gamma_0 + N\gamma_1)/2}{((\gamma_0 + N\gamma_1)/2)^2 + (\omega - \omega_0 + (2n-N)\omega_1)^2}$ is a Lorentzian and $a_1 = \exp(-\hbar\omega_1/(k_B T))$ is the Boltzmann factor of this specific mode.

Equation (3) was used to analyze the experimental data. It has to be mentioned that only one normalization factor was used to adjust the calculated intensities to the experimental data at all temperatures. In order to avoid an averaging over the scattering vectors $\vec{k}$ we used the following approximation: Our geometry determined an unresolved momentum transfer k between 2.6 and 14.4 Å^{-1}. The probability of the momentum transfer is almost constant between 4 and 14 Å^{-1}. Considering the absorption of the incoming beam in the sample one has a smoothly decreasing distribution falling from a maximum at 5 Å^{-1} to half the maximum at 14 Å^{-1}. The scattered intensity in this momentum range was taken as incoherent. The intensity weighted mean scattering angle between $\vec{k}_0$ and $\vec{k}_1$ was $2\vartheta = 34°$, corresponding to a momentum transfer of 4.3 Å^{-1}. This angle was then used in the data analysis to obtain the scattering vector $\vec{k}$.

The frequency of the single vibrational mode was adjusted to $\hbar\omega_1 = 12.4$ meV. The Lorentzian line widths were $\gamma_0 = 4.4$ meV for the central elastic line and $\gamma_1 = 14$ meV for the off center inelastic lines. The latter value was assumed to reduce an artificial structure in the wings of the scattered intensity which is due to the use of a single vibrational mode. This artefact will disappear with the use of many vibrational modes from a normal mode analysis.

For an evaluation of the temperature dependence of the scattered intensity one needs the slope of the mean square fluctuation with temperature. For all temperatures we use $\langle R_1^2\rangle/T = 8.7 \cdot 10^{-4}$ Å^2/K. This value was obtained from the normal mode analysis of myoglobin (Melchers et al. 1996) by averaging over all atoms of the myoglobin plus the 170 water molecules from the crystal structure. The relative contribution of the water molecules was enlarged by the factor 400/170 to account for the total water content of the sample.

Figure 2 shows that the model provides a good approximation to the experimental data at all temperatures although it assumes only a single vibrational mode. The spectral shapes are well described using the same average slope $\langle R_1^2\rangle/TT$ obtained from the normal mode analysis.

The total area of the calculated spectrum is independent of temperature for the spectra at T = 62 K, 124 K and 189 K. We believe that the additional intensity of 11% at T = 300 K comes from the readjustment of the equipment. A constant area shows that our measurement records all types of scattering processes, including elastic, quasielastic and inelastic scattering. The harmonic approximation of the rms fluctuations is sufficient to explain the temperature dependence of the spectra. This shows that this type of motion is present in the whole temperature regime. This is in agreement with our previous interpretation of the Mössbauer absorption experiments on myoglobin crystals (Parak et al. 1982, Knapp et al. 1982). However, at first sight our present data seem not to be able to explain the additional loss of intensity of the Mössbauer absorption spectra between 200 K and 300 K yielding a mean square displacement of the iron which increases much more than linearly with temperature. For an understanding of this effect one has to have in mind that the energy resolution of Mössbauer absorption spectroscopy and of the present X-ray scattering experiment differs by nearly 6 orders of magnitudes. The protein specific dynamcis which become measurable above about 200 K are probably due to diffusive motions in a restricted space. The intensity missing in the elastic line may be found in the broad quasielastic lines seen in the Mössbauer absorption experiments. In our X-ray scattering experiments, presented here, these quasielastic lines cannot be resolved. Our central peak counts for both elastic and quasielastic scattering.

The spectral shape of our data can be resonably well described at all temperatures by an harmonic approximation. Together with the results of Mössbauer absorption experiments this is a strong hint that protein dynamics at physiological temperatures can be understood as a superposition of two types of motion. In addition to the more or less harmonic molecular vibrations, diffusive structural changes take place close to physiological temperatures and these can be understood as jumps between conformational substates of the molecule.

The work was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie under contract 05 643 WOC and the DFG (Graduate College FU-Berlin).

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