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Determination of the phonon spectrum of iron in myoglobin using inelastic X-ray scattering of synchrotron radiation

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Dynamic features of a solid reveal themselves in the photon density spectrum. In the case of more or less simple crystals such as copper (Nicklow et al. 1967) or naphthalene (Natkaniec et al. 1980) it was possible to resolve the dispersion relation in the different crystallographic directions in coherent neutron scattering experiments, and, in this way, to obtain the phonon spectrum. In biomolecules the problem is more complicated. The main difficulty in such experiments is the large incoherent scattering cross section of the hydrogen atom. For a coherent neutron scattering experiment relatively large protein cystals of completely deuterated material have to be available. At present this requirement is a bottleneck for such experiments. Nevertheless, neutron scattering is a successful tool to determine phonon spectra of biomolecules. Using the incoherent scattering of the hydrogen, phonon spectra of myoglobin (Cusack and Doster 1990) and BPTI (Cusack et al. 1988) have been determined. However, such experiments are sensitive to the motions of the hydrogens only. Apart from the modes of motions of the backbone coupled to the hydrogen, motions such as the rotation of the methyl groups also contribute. In order to study the influence of protein dynamics on protein function it is desirable to have methods which are sensitive to the non-hydrogen atoms of a protein.

Recently we applied a new method (Chumakov et al. 1996), implemented at the nuclear resonance beamline BL11 at ESRF (Rüffer and Chumakov 1996), to the investigation of protein dynamics using inelastic X-ray scattering of synchrotron radiation which was analysed by the Mössbauer effect (Achterhold et al. 1996). In this method a monochromator system selects X-rays in the region of 14.4 keV with a full width at half maximum of 4.4 meV from the white synchrotron radiation. This radiation, which is close to the Mössbauer resonance condition in ^{57}Fe, is scattered by the sample. If the scattered radiation gains or loses energy by phonon interaction it can be shifted into the energy window of the Mössbauer effect of ^{57}Fe. This part of the radiation can be detected by using a ^{57}Fe foil as analyser together with a time resolving detector. As a result a phonon spectrum of wet metmyoglobin powder was obtained (Achterhold et al. 1996) where the motions of all atoms of the sample contribute. Besides hydrogen atoms a protein consists mainly of N-, C- and O-atoms. Since the scattering of the atoms is weighted by the number of their electrons this method essentially gives information on the modes of motions of the N-, C- and O-atoms. In the following we describe an experiment where the set-up is modified in a way that we are able to determine selectively modes of motions which couple to the iron atom (Seto et al. 1995, Sturhahn et al. 1995). Our sample consisted of a large number of small deoxymyoglobin single-crystals. The heme iron was replaced by the Mössbauer isotope ^{57}Fe. The experiment was performed at room temperature at the beamline BL11 of the ESRF.

The experimental set-up is shown in Fig. 1. X-rays of 14.4 keV tuneable in the range of ±100 meV are selected from the synchrotron radiation after an undulator of 23 mm period by a double Si(111) monochromator and a nested monochromator (Ishikawa et al. 1992) using the reflections (422) and (975) of silicon. This radiation with the width of 4.4 meV irradiates the myoglobin sample. The scattered radiation is collected in the avalanche photodiode (Baron 1995) APD1 while the transmitted radiation is detected in the avalanche photodiode APD2. The photodiode APD1 was adjusted in a way to obtain as much scattered radiation as possible. The photodiode had no energy selectively. Therefore, besides the scattered X-rays, the 6.4 keV

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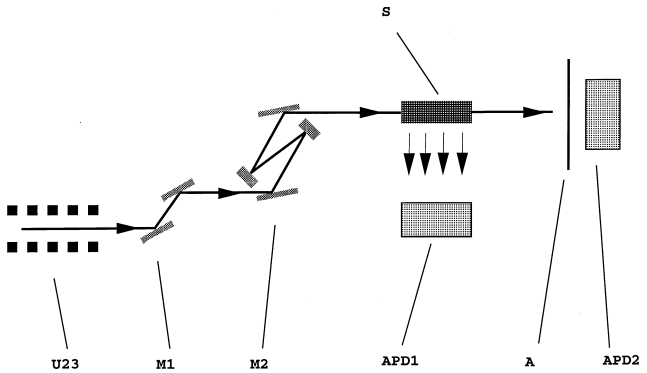


Fig. 1 Set-up for the experiment: U23 – undulator with a 23 mm period, M1–Si(111) double crystal monochromator, M2–Si(422) and Si(975) high resolution monochromator, APD1 and APD2 – avalanche photo diodes, S – myoglobin sample, A – ^{57}Fe metal Mössbauer analyzer foil inserted in the direct beam for energy reference and resolution determination

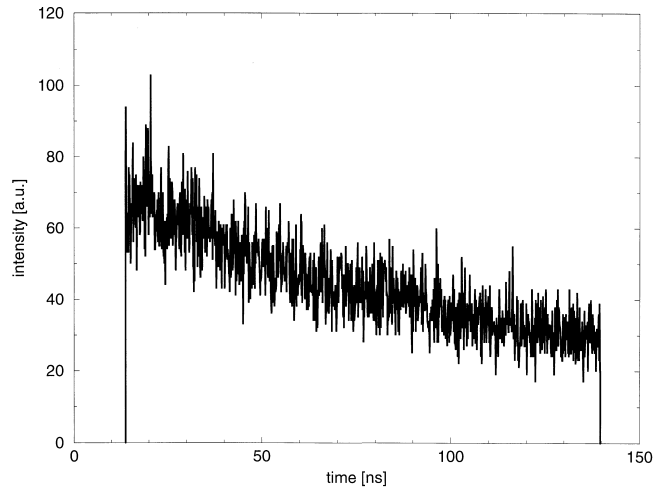


Fig. 2 Time spectrum of a superposition of the incoherent nuclear scattering of ^{57}Fe and the 6.4 keV K_α X-rays of iron emitted after Mössbauer absorption in ^{57}Fe in myoglobin at a particular incoming energy. An integration between 12 and 150 ns yields one intensity value shown in Fig. 3

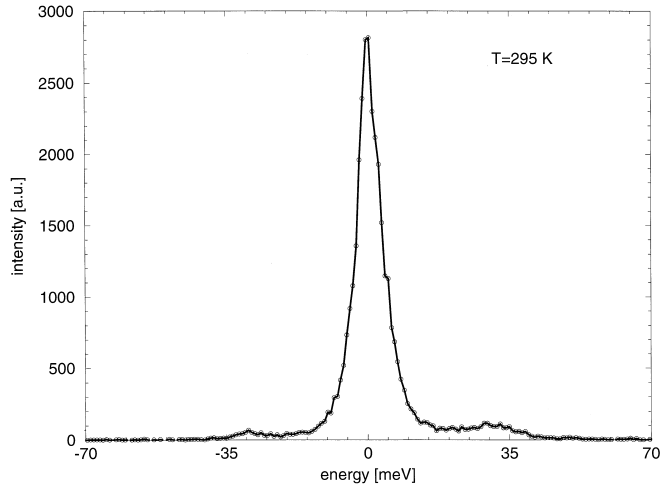


Fig. 3 Intensity spectrum of phonon modes coupling to the ^{57}Fe in myoglobin as a function of the energy difference of the synchrotron beam from the Mössbauer resonance. Measurement at room temperature

K_α radiation of iron was also detected. This radiation is emitted if a Mössbauer absorption at the ^{57}Fe nucleus is followed by an internal conversion. The detector covered a solid angle of 2.5·1.9 sterad. In our experiment the storage ring worked in the 16 bunch mode yielding every 176 ns an X-ray flash with a length of ~100 ps. Although prompt scattering overloads the avalanche diodes they were able to resolve the time structure of the scattered radiation from 10 ns after the prompt pulse on.

The time resolution of the avalanche diodes is used to separate elastic Rayleigh scattering by the electrons of the myoglobin molecules from the time delayed Mössbauer scattering of the ^{57}Fe in the heme group. The prompt process is finished immediately after the X-ray pulse of ~100 ps. During this time no data collection occurs. As shown in Fig. 2 there is a time delayed signal in the APD1 starting after the death time of the diode. This signal comes from the following process: modes of motions which couple to the heme iron of the myoglobin can add or subtract the energy which is necessary in order to get Mössbauer absorption in the iron nucleus. The re-emission occurs with a time delay which is characterised by the average lifetime of the 14.4 keV level of the ^{57}Fe nuclei ($\tau_N = 141$ ns). To optimise the counting rate the discriminator thresholds were set to detect the 14.4 keV Mössbauer radiation as well as the 6.4 keV fluorescence X-rays from internal conversion. Note, that the time structure of the time delayed scattering is a simple exponential with a decay time τ_N. Single-nucleus quantum beats due to the quadrupole splitting of the excited nuclear state of deoxymyoglobin are not seen because about 98% of the detected intensity is due to the 6.4 keV FeK_α radiation. Integration over the time spectrum of the delayed intensity gives one intensity in the energy spectrum. Figure 3 shows the scattered intensity as a function of the incoming energy. The energy scale is chosen in such a way that the Mössbauer transition of the ^{57}Fe nucleus is taken as reference energy. The spectrum shows clear features. Note that the maximum of the phonon density lies close to the well known energy (27.5 meV) of the iron histidine vibrations in deoxymyoglobin as determined by Raman spectroscopy (Argade et al. 1984). The temperature dependence of this spectrum and a detailed discussion using the normal mode analysis as described by Melchers et al. (1996) will be given elsewhere. A comparison of this experiment with the results of our previous inelastic X-ray scattering experiment (Achterhold et al. 1996) shows a dramatic difference of the two spectra. This reflects the fact that out of all the modes of motion of the entire molecule only few are coupling to the iron. The shape of the energy spectrum is therefore changed completely.

The previous experiments in which we obtained the total inelastic X-ray scattering were performed on a wet powder of metmyoglobin. The present experiment was done on

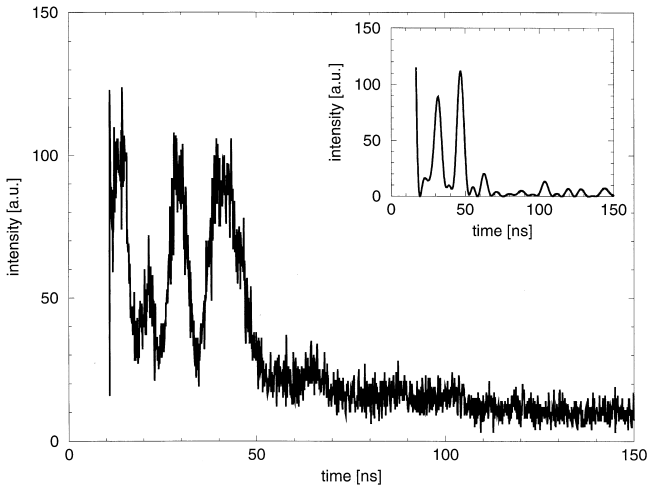
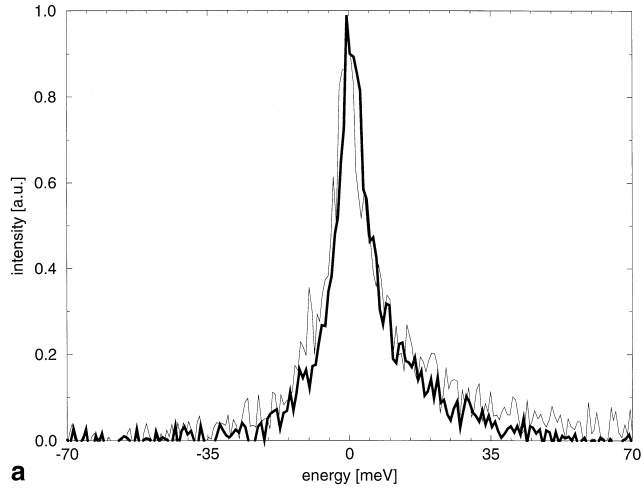


Fig. 4 Superposition of the incoherent nuclear scattering of ^{57}Fe in myoglobin and the nuclear forward scattering of an iron metal foil put before APD1 as a function of time. *Inset*: Calculated time spectrum of the iron metal foil before APD1



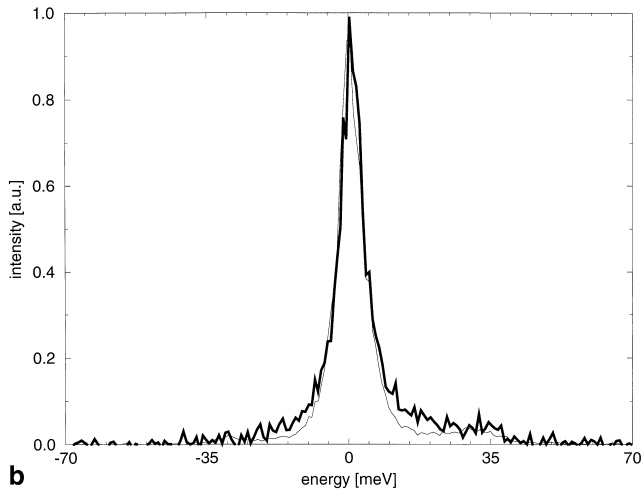


Fig. 5 **a** Thick line: Energy spectrum originating mainly from inelastic Rayleigh scattering of synchrotron radiation on the myoglobin sample detected in the 12 to 50 ns time window of the superposition time spectrum of Fig. 4b. *Thin line:* Metmyoglobin powder spectrum taken from (Achterhold et al. 1996). **b** Thick line: Energy spectrum originating mainly from inelastic nuclear scattering by ^{57}Fe in myoglobin detected in the 50 to 150 ns time window of the superposition time spectrum of Fig. 4 compared with the spectrum of Fig. 3 (*thin line*). The statistics are much worse than in Fig. 3 since the intensity coming from the 6.4 keV K_α radiation of the iron is missing

single crystals of deoxymyoglobin. There remains the question of whether it is valid to compare these two samples. In order to get the total inelastic X-ray scattering on the present sample we use the following trick. We put an iron foil before the APD1. Inelastically scattered radiation gaining or losing the energy which is necessary to fall into the energy window of Mössbauer absorption of ^{57}Fe yields a time delayed radiation with the structure shown in the inset of Fig. 4. The structure is only determined by the hyperfine interaction within the iron foil used as analyser. In this experiment the 6.4 keV fluorescence X-rays from internal conversion within the analyser were eliminated using a 300 μm aluminium foil. Radiation which is inelastically scattered at the heme iron yields in the mean the natural decay as shown in Fig. 2, dominated now by the 14.4 keV radiation. If we perform our scattering experiment with the ^{57}Fe enriched myoglobin crystals and the ^{57}Fe iron foil before the avalanche diodes we obtain a weighted superposition of both time structures. As shown in Fig. 4 the collected intensity is dominated by the quantum beats of the iron foil in the time region between 12 and 50 ns revealing the origin from inelastic Rayleigh scattering while at later times the intensity mainly comes from the scattering at the iron nucleus in myoglobin. By setting time windows we are able to separate the two processes in one experiment. Figure 5a shows the result if we use only the quanta with the time delay between 12 and 50 ns. The obtained energy spectrum is nearly identical with that of inelastic Rayleigh scattering (Achterhold et al. 1996). Figure 5b compares the spectrum with the time delay between 50 and 150 ns with the spectrum of Fig. 3 showing the dominating contribution of the ^{57}Fe of myoglobin in this time regime.

Compared with the huge number of publications dealing with the dynamic properties of crystals of inorganic and organic material the study of the dynamics of biomolecules is still at the beginning. In addition to the spectroscopic methods such as optical- (Cupane et al. 1995), infrared- (Steinbach et al. 1990) or Mössbauer spectroscopy (Parak and Achterhold 1995) there now exist three different types of scattering experiments yielding complementary information on phonon density spectra. Incoherent neutron scattering gives information on modes coupling to hydrogens. The Rayleigh scattering of synchrotron radiation allows one to get information on the motions of all atoms within the molecule. Both experiments give averages. One has to use models in order to obtain information on the motions of the individual atoms. The experiment described in this letter adds a new possibility. It should be noted that Harami et al. (1996) performed a very similar

experiment on HbCO solution. However, in contrast to our experiment the diffusion of the HbCO molecules washed out the details of the phonon spectrum.

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