Proteins in action: the physics of structural fluctuations and conformational changes
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Structural dynamics is essential for the biological function of proteins. Results from new experimental techniques should be compared with those from previous experiments in order to obtain a consistent picture of the physics of intramolecular fluctuations and conformational changes. The high intensity and time structure of synchrotron radiation have made possible time-resolved X-ray structure analysis and the determination of phonon density spectra through the Mössbauer effect. By combining results from Mössbauer absorption spectroscopy, incoherent neutron scattering, low-temperature crystallography and optical spectroscopy, a physical picture of protein dynamics emerges.

Introduction
Proteins are essential components of life. They resemble tiny engines on the scale of nanometres. Examining a static picture of an engine, as conventional X-ray structure analysis does in the case of a protein, may give only a limited understanding of its function. Engines should be studied in action; one has to investigate protein dynamics. This can be done at different levels. At unphysiologically low temperatures, the molecules are in a ‘stand-by’ state; functional action is not activated. Above the so-called dynamical transition temperature, T_c, protein-specific motions occur, allowing the molecular engine to fulfill its function.

Studies of an engine in action should concentrate on one single copy. With proteins, this is possible only if single-molecule methods are applied. In general, investigations are performed on a large ensemble of typically 10^{15} molecules to get measurable signals. All machines should run synchronously to produce a movie of the motions, otherwise one obtains averages over a large number of motional states. Depending on the experiments, a selective sensitivity to motions within a certain time window can be obtained. In the ensemble average, all processes are seen that fall into this time window, determined by the energy resolution of the experiment.

Systematic investigations of dynamics have been performed mainly on myoglobin, which plays a special role as a paradigm of protein dynamics [1]. In the following, results obtained using several experimental methods are compared for myoglobin in order to get a consistent description of the dynamic properties of a protein. In order to understand the biologically important mechanism of conformational changes, this review also emphasizes its prerequisites, such as harmonic vibrations, structural distributions and structural fluctuations.

Solid-state physics of proteins
In the stand-by state of a protein (below T_c), the dynamics is characterized by the density of phonon states, as in solids. The phonons act as a ‘thermal bath’, which supplies the energy for all processes of protein dynamics. For a long time, only the incoherent neutron scattering was used to determine the density of states. However, this method is sensitive merely to phonons coupling to the hydrogen atoms. In iron-containing proteins, the use of synchrotron radiation now makes it possible to determine the density of states of phonons coupling to the iron, using the so-called ‘phonon-assisted Mössbauer effect’ [2–4,5**,6].

Monochromatised synchrotron radiation of energy E_{syn} with an energy width of typically 1 meV, can be varied by ± 80 meV with respect to the nuclear resonance energy (E_γ = 14.4 keV) of the isotope ^{57}Fe. Even when E_γ and E_{syn} do not overlap, nuclear resonance scattering occurs if the energy difference (ε) is balanced by phonons coupling to the iron. Then, the intensity of nuclear resonance scattering is a measure of the phonon density at ε. Figure 1 shows the phonon density as a function of energy at the haem iron in myoglobin using low-temperature experiments [5**]. In the experiment, the intramolecular phonons (ε > 1 meV) cover a time window from about 0.1 to 0.01 ps. From the density of states, it is possible to calculate the mean square displacements of the iron. Figure 2 shows how the mean square displacements depend on the energy of the contributing phonons. Some deviation from the linear increase of the mean square displacements with temperature occurs only at room temperature. There is no indication of a dynamical transition temperature. In a later discussion, this fact is used to identify the physical meaning of T_c.
The density of phonon states as a function of energy (ε). Solid line, metmyoglobin; dashed line, CO-ligated myoglobin; dotted line, deoxymyoglobin. Note that, below 1 meV, the density of states determined by incoherent neutron scattering is the same as that shown here, proving that the density stems from acoustic modes.

**Protein-specific peculiarities at low temperatures**

Even below Tc, optical spectroscopy and X-ray structure analysis reveal typical features of proteins, which may be reminiscent of glass. Protein molecules have no well-defined energy minimum. This has been concluded from the rebinding kinetics of CO after photolysis of CO-ligated myoglobin [7,8]. Hole burning experiments prove the heterogeneity of an ensemble [9]. On the timescale of days, some dynamics is still present even at T = 4.2K. ‘Structural distributions’ of proteins were confirmed by the determination of individual mean square displacements, <x²>, of the nonhydrogen atoms obtained from the Debye–Waller factor of X-ray structure analysis [10]. Recently, it became possible to separate protein internal structural distributions from displacements of the whole molecule in the crystal. Extrapolating the linear decrease with temperature of the mean square displacements to T = 0K yields a zero point structural distribution [11*]. This could be seen as an analogy to the zero point entropy of ice.

**Conformational changes**

Protein molecules can adopt different conformations. Even simple proteins such as myoglobin change conformation if small molecules (e.g., CO or O₂) bind. Without such a ligand, myoglobin is in the so-called t conformation; following ligation with CO or O₂, it adopts the so-called r conformation. The suppression of conformational changes results in the loss of biological activity. Recently, the conformational change of the L29W mutant of sperm whale myoglobin has been investigated [12]. This mutant performs the same function as the wild type, but with strongly reduced rates. To trigger the conformational change, the CO ligand is photolyzed by illumination with laser light. Structures were determined at 105K after different thermal treatment of the sample. The irradiation started either below 180K or above 180K. In both cases, the temperature was then reduced to 105K during illumination. For comparison, the structure of the CO-ligated L29W mutant was also determined in the dark. Figure 3 shows the results. Illumination below 180K photolyzed the CO molecule, which was found docked close to the tryptophan. Illumination above 180K also “flashed off” the CO molecule. In this case, however, the change in the coordination of the iron is accompanied by a large conformational change on the distal side. In the frozen samples, only the CO molecule may travel within the protein moiety, whereas at temperatures above 180K conformational relaxations can occur — the protein is functionally active.

**Structural fluctuations — the lubricant for functional motions**

Structural fluctuations can be probed by dynamic mean square displacements, <x²>, of atoms in the molecule. Mössbauer absorption spectroscopy labels dynamical displacements at the iron and incoherent neutron scattering
averages over the motions of all hydrogen atoms (\(^1\)H) in the sample \([13^{*}]/C15/C15\). Some results are shown in Figure 2. Excluding zero point vibrations, the \(<x^2>\) values increase linearly with temperature below 180K. The \(<x^2>\) values, obtained by Mössbauer absorption spectroscopy, are in good agreement with those calculated from the phonon density spectrum integrating to \(\varepsilon = 80\) meV. A strong increase in the slope of \(<x^2>\) versus \(T\) is obtained above \(T = 180\)K using Mössbauer absorption experiments and

Mössbauer absorption spectroscopy, as well as incoherent neutron scattering, shows a change of the spectral shape at \(T_c\) \([13^{*}]/C15/C15\). This is characteristic of quasi-elastic processes and indicates diffusion-like motions. The two experimental methods have complementary time windows; incoherent neutron scattering measures only processes faster than 100 ps, whereas \(^{57}\)Fe absorption spectroscopy is sensitive to motions faster than 100 ns. From the shape of the Mössbauer absorption spectrum, one can conclude that \(<x^2>\) is caused by motions occurring between 1 ns and 300 ps at room temperature. Recent neutron structure analysis of myoglobin \([14]\), together with published results of incoherent neutron scattering \([15]\), indicates that, above \(T_c\), neutrons label essentially the protein-specific motions of the sidechains \([13^{*},16]\), whereas Mössbauer spectroscopy measures preferentially the slow motions of the backbone coupling to the haem iron.

Incoherent neutron scattering has also been used to investigate the dynamics of several other biological systems \([17^{*},18–20]\). Mössbauer absorption spectroscopy and incoherent neutron scattering indicate a characteristic temperature, \(T_c\), which is a loosely defined border between a stand-by state of the protein and a functionally active state. However, such separation is not seen in the phonon-assisted Mössbauer effect, indicating a stand-by state up to room temperature. For an understanding of this apparent discrepancy, one has to take into account the different energy resolutions of the experiments connected directly with the time resolutions. The energy resolution of the phonon-assisted Mössbauer effect is about 1 meV, corresponding to a time resolution of \(\tau = 1/\omega = 0.7\) ps. Only processes with a faster characteristic time contribute. These are essentially the optical phonons. Mössbauer absorption spectroscopy on \(^{57}\)Fe has an energy resolution of about \(10^{-6}\) meV and a time resolution of 100 ns. The incoherent neutron scattering experiments were sensitive to motions faster 80 ps. The comparison tells us that the protein-specific motions activated above \(T_c\) occur on a very slow timescale in the region between 1 ps and 100 ns. Note that the above arguments are valid only for incoherent processes.

### Physical models of protein-specific dynamics

CO-ligated myoglobin was one of the first molecules to be investigated by time-resolved X-ray structure analysis \([21,22,23^{*}]\). The experiments were carried out at room temperature. The dissociation of the CO molecule is
triggered by a short laser pulse. The corresponding change of the electron density map is followed from 1 ns to 1 ms. At 1 ns, 20% of the CO molecules have already reached a docking place close to the location of the water molecule in deoxygenymyoglobin. In agreement with previous investigations, the iron is out of the haem plane, which is domed. Moreover, motions towards the deoxy structure of parts of the backbone close to the haem centre are indicated. This partial change of conformation within 1 ns is in good agreement with the characteristic time of protein-specific fluctuations seen by Mössbauer absorption spectroscopy. However, one should not forget that many fluctuations are necessary to assist a conformational change. Also, the X-ray structure at 1 ns after the maximum of the laser flash actually averages over 4.5 ns of illumination because of the width of the laser pulse. Within the limits of experimental imprecision, the agreement of the experiments is very good.

After about 100 ns, the photolyzed CO reaches the Xe1 cavity. Obviously, a large number of diffusion-like motions are necessary to open a diffusion path in a stepwise manner. It should be mentioned that the path of the CO molecule in myoglobin and the time dependence have been investigated by many authors [24–29]. It should be noted that, in this review, the very fast processes (e.g. movement of the iron, doming of the haem) are not discussed. A recent investigation of these primary effects, also present below Tc, is found in [30*].

The results of a time-resolved X-ray structure analysis of a protein are often presented as a movie. One has to keep in mind some peculiarities of the experiments. The path from one conformation to the other is different for each individual molecule in the crystal. Therefore, the experiments cannot visualise the atomic trajectories by which one molecule passes from one state to the next. The movies show the time dependence of the population and depopulation of intermediate states occupied by a reasonable fraction of the molecules. Intermediate states can often be trapped by freezing, making conventional X-ray analysis possible. However, time-resolved X-ray structure analysis can follow the occupation of intermediate states in real time and yields the reaction kinetics of conformational changes at the atomic level. This has already been clearly demonstrated [31**].

Some years ago, Miller and co-workers [32,33] performed grating spectroscopy experiments to study the time dependence of the r → t conformational change in myoglobin. CO is flashed off by a laser pulse. This causes density changes by thermal expansion and conformational changes of the volume, generating standing acoustic waves in the water environment of the protein. From the extremely fast rise time of the density change induced by conformational changes after CO photolysis, it is concluded that large-scale global protein motions occur on a picosecond timescale. A comparison with other experiments raises some questions. Earlier spectroscopic measurements indicate structural relaxations from 2 ps to 56 µs [34]. Time-resolved X-ray structure analysis shows no density changes on the surface of the molecules 1 ns after CO photolysis [23**], although a comparison with myoglobin and CO-ligated myoglobin structures, determined at high resolution, predicts differences [35]. Is the sound wave created by the motions in the haem region enough to explain the effect? Could a strong vibration mode be excited without enforcing the r → t transition?

Recently, the temperature dependence of some reaction constants (e.g. the jumping of CO from the Xe1 position into water) was compared with the dielectric fluctuations of the surrounding solvent [36]. It was concluded that solvent fluctuations dominate some part of protein dynamics and control function. The fluctuation of the water molecules provides the enthalpy that the protein needs to go from the rigid to the flexible state. The protein, together with its hydration shell, determines the entropy, which controls the motions in the flexible state. For example, it is responsible for the opening and closing of channels. Also, the diffusion-like motions found in Mössbauer absorption spectroscopy were explained by entropy-controlled motions in the flexible state, whereas the transition from the rigid to the flexible state is enthalpy controlled.

Conclusions
The investigation of protein dynamics will be a fascinating topic for the future. The physical pictures discussed above are mainly based on one small helical protein. It is not known how well the physics can be generalized to β-sheet proteins or even to much larger enzymatic proteins. Nevertheless, a physical concept has emerged. Below a characteristic temperature, Tc, the molecules are frozen in slightly different structures — conformational substates — belonging functionally to the same conformation. The energy of the thermal bath, including the environment of the molecules, is too low to allow transition between substates in functionally relevant times. The molecules are in a rigid state. Above Tc, the molecules can reach a ‘flexible’ state, in which diffusion-like motions of molecular segments render possible the exploration of different substates. The fluctuation of molecules in the flexible state is necessary for possible conformational changes. It also supports the travelling of CO in the protein. To get a very rough image of the rigid and flexible states, one may consider the structure of water. Several water molecules are locked together in an ice-like structure for some time. The breaking of only a few hydrogen bridges makes this cluster liquid until a new arrangement of the hydrogen bridges makes it ice-like again. Figure 4 summarizes some essential features.
Simplified scheme for the conformational change of myoglobin upon ligation of CO (not including very fast processes such as the motion of iron and the doming of the haem). Parabolas indicate flexible states, sticks represent rigid states (conformational substates). The drawing has one spatial coordinate instead of three, labelling the motion at an arbitrary point in the molecule (a complete representation should include 3N-6 coordinates for a molecule with N atoms). A picture with two spatial coordinates is shown as an inset at bottom right. Mb and MbCO are equilibrium states of deoxymyoglobin and CO-ligated myoglobin, respectively. E_{CO} is the binding energy of CO and \( \lambda \) is the energy of a structural rearrangement. Except for small changes around the haem iron, MbCO and Mb* have the same structure, as do Mb and Mb’CO. Flashing off the CO brings MbCO to Mb*. If this occurs below \( T_c \), the molecules are rapidly trapped in the rigid state of Mb*. They cannot perform diffusion-like motions. Above \( T_c \), the diffusion-like motion in the flexible state results in structural relaxation to Mb (on the nanosecond timescale). Binding CO brings Mb to Mb’CO. Above \( T_c \), structural fluctuation in the flexible state of Mb’CO allows structural relaxation to MbCO. In this scheme, all intermediate states between Mb* and Mb (Mb’CO and MbCO) are omitted. The transition MbCO \( \rightarrow \) Mb* and Mb \( \rightarrow \) Mb’CO is fast compared to Mb* \( \rightarrow \) Mb and Mb’CO \( \rightarrow \) MbCO.

**Update**

Recently, two publications appeared describing the use of time-resolved X-ray structure analysis for the investigation of the conformational changes of myoglobin after photolysis of a CO ligand. In both cases, myoglobin mutants were used, slowing down [38] or accelerating [39] the binding rate of CO. In [38], it was shown that processes in the vicinity of the haem, such as dissociation of CO, motion of the iron and tilting of the haem, are fully developed 3 ns after CO photolysis. However, a few hundred nanoseconds are required for the globin to fully respond to the fast structural changes of the haem. The improved time resolution of the experiment described in [39] yielded additional information. In this mutant, functionally important sidechain motions close to the haem were observed several picoseconds after CO photolysis. From 316 ps to 3.16, the structural changes are largely confined to the vicinity of the CO-binding site.

Both experiments are in good agreement with the physical model of protein dynamics discussed in this review.

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**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


This paper describes in detail the experiments and theory behind the determination of the density of phonon states coupling to the haem iron in myoglobin. The calculation of mean square displacements of the iron from the density of states is explained.


X-ray structures of myoglobin determined in a wide temperature range (40K to room temperature) were analysed by ‘normal mode refinement’. This allowed the separation of intermolecular (translation, libration and screwing of the molecule as a whole) and intramolecular modes of motions. Only the intramolecular modes are of interest for protein dynamics.
of protein dynamics by Mo looking at this detailed review. The literature concerning the investigation of protein dynamics by Mössbauer absorption spectroscopy is cited since 1971.


The authors present a model for understanding protein-specific motions of hydrogen atoms. They show that dynamic transition temperatures and protein-specific motions are not limited to myoglobin, but can also be seen in completely different proteins.


An interesting analysis of the out-of-plane motion of the iron after fast photolysis of CO in very short times. The conclusions concerning the structural transitions and the release of the CO into the solvent are less convincing.


This paper clearly demonstrates the excellent perspectives of time-resolved X-ray structure analysis.


