Using synchrotrons and XFELs for time-resolved X-ray crystallography and solution scattering experiments on biomolecules

Matteo Levantino[1], Briony A Yorke[2], Diana CF Monteiro[3], Marco Cammarata[4] and Arwen R Pearson[2]

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Time-resolved structural information is key to understand the mechanism of biological processes, such as catalysis and signalling. Recent developments in X-ray sources as well as data collection and analysis methods are making routine time-resolved X-ray crystallography and solution scattering experiments a real possibility for structural biologists. Here we review the information that can be obtained from these techniques and discuss the considerations that must be taken into account when designing a time-resolved experiment.

Addresses
[1] Department of Physics and Chemistry, University of Palermo, Palermo 90128, Italy
[2] Hamburg Centre for Ultrafast Imaging & Institute of Nanostructure and Solid State Physics, University of Hamburg, Hamburg 22607, Germany
[3] Astbury Centre for Structural Molecular Biology & School of Chemistry, University of Leeds, Leeds LS2 9JT, UK
[4] Department of Physics, UMR UR1-CNRS 6251, University of Rennes 1, Rennes 35042, France

Corresponding author: Pearson, Arwen R (arwen.pearson@cfel.de)

Introduction

The relationship between the structure of proteins and their function is determined and controlled by their dynamic properties. From the process of polypeptide translation by the ribosome, through folding to form the functional protein and finally as the protein undergoes its particular function in the cell or across the cell membrane, molecular motions play a crucial role. A complete understanding of protein function therefore requires experiments that are able to probe both structural and dynamic properties. X-ray crystallography and solution scattering are routinely used methods to examine molecular structures (Figure 1). However, when used in a time-resolved manner, these methods can also enable the observation of functionally related structural changes

and dynamics over a broad range of spatial and temporal resolutions.

The time-scales of biologically relevant processes range from fs (10^{-15} s) to hours and so the design of a time-resolved experiment must be matched to the time-scale of the process of interest (Figure 2). For example, while an enzymologist might wish to focus on early steps in a reaction mechanism occurring on fs–ns time-scales, a molecular biologist might be interested in the large scale conformational rearrangements associated with ligand binding or signal transduction that occur in μs–ms. Recent developments in X-ray sources, beamline instrumentation and data collection methodologies now provide structural biologists with a suite of experimental tools to simultaneously probe biomolecular structure and dynamics.

X-ray methods to study biomolecular structure and dynamics

Time-resolved X-ray crystallography is the technique that is currently able to provide the greatest spatial and temporal resolution over the whole structure of a protein. However, it is limited by the requirement for well diffracting protein crystals. Although great advances have been made in this field, crystallization remains an art rather than an exact science. Excitingly, the possibility of performing X-ray diffraction experiments on *in vivo* grown protein microcrystals has been recently demonstrated [1,2•]. However, major protein classes such as membrane proteins and intrinsically disordered proteins, both key players in the life of the cell, are difficult or impossible to crystallize. This challenge is compounded by the delicate nature of protein crystals. Large conformational changes can either destroy the crystalline lattice, making X-ray data impossible to collect, or be restricted by the crystal packing, preventing reaction progression in the crystal.

An alternative approach to study the dynamics of biological macromolecules is to monitor the time evolution of X-ray scattering by liquid solutions of the biomolecules of interest. This has recently emerged as a powerful method to investigate relevant biological reactions on systems that are either not easy to crystallize or undergo large scale conformational changes that cannot take place within a crystalline environment, such as quaternary structure
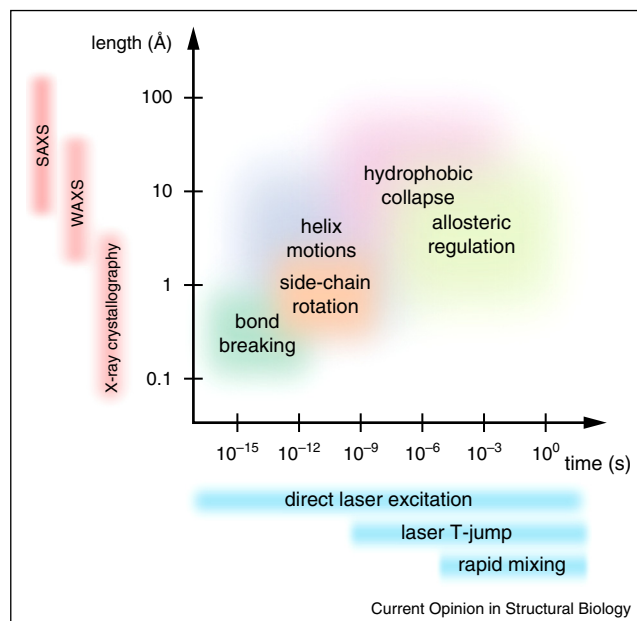
Figure 1
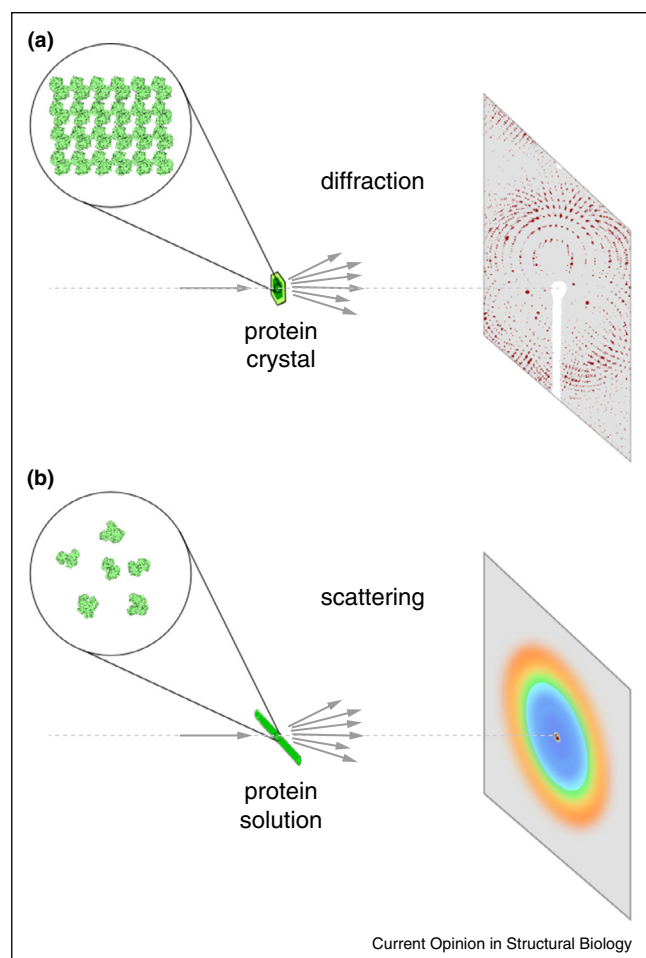
Time-resolved X-ray diffraction from protein crystals and scattering from protein solutions provide complementary information. Crystallography can yield atomic resolution information about the whole molecule, but requires that the crystal remains intact during the reaction. Solution scattering is extremely sensitive to structural changes with the advantage that crystals are not required. However, associating the scattering changes with specific structural rearrangements remains difficult.

Figure 2

Conformational changes of biomolecules can be triggered with different methods (i.e. direct laser excitation, laser T-jump and rapid mixing) and monitored with time-resolved X-ray crystallography, wide-angle X-ray scattering (WAXS) or small-angle X-ray scattering (SAXS). The experimental technique and the triggering method must be matched with the time-scale and type of structural change to be investigated. Although crystallography can — in principle — be used to detect conformational changes extending up to tens-of-Angstroms, often only relatively small motions can be accommodated by the crystalline environment and thus be observed in a time-resolved experiment.
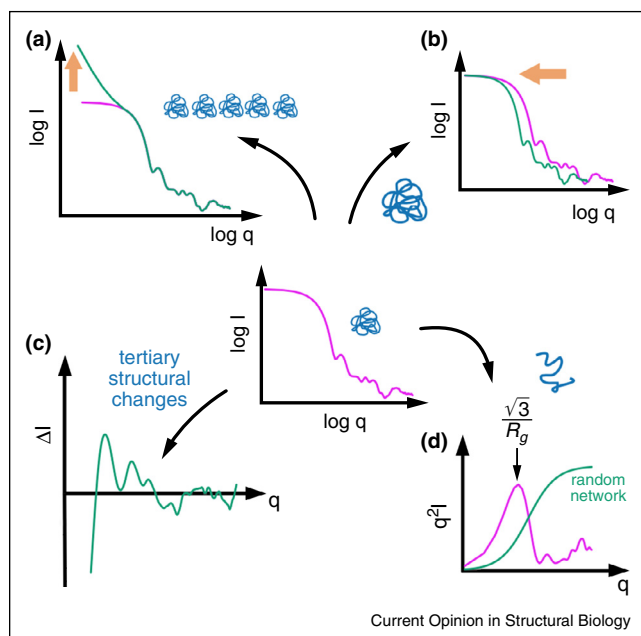
Designing a time-resolved experiment

When planning a time-resolved experiment a number of factors must be taken into consideration. First, the time-scales of interest must be identified as these will define the X-ray source and experimental protocol required. Once the decision as to type of experiment and time-resolution required has been reached then the question of reaction initiation must be addressed.

Choice of source and experimental protocol

In any time-resolved experiment, the key consideration is signal to noise (S/N). The fundamental time-resolution of the experiment is determined by the time it takes to deliver enough photons onto the detector for a measurable signal to be obtained. How fast the required number of photons can be delivered depends on the brilliance of the X-ray source.

Third generation synchrotron radiation sources are able to provide X-ray pulses as short as ~100 ps and, by using polychromatic Laue radiation, can deliver 10^{10} photons per pulse at the sample. Such X-ray photon flux is sufficient to perform time-resolved experiments at time-scales

rearrangements, macromolecular folding or protein aggregation. The ability to investigate such a large variety of biologically relevant processes comes at the expense of spatial resolution and unbiased retrieval of structural information. The X-ray scattering pattern from an ensemble of randomly oriented and unequally spaced macromolecules lacks the characteristic Bragg peaks of crystalline samples (Figure 1). Nevertheless, changes in the overall dimension/mass can be readily measured in the SAXS (small-angle X-ray scattering) region of the scattering pattern while higher resolution information (relative position of different subunits, domains, or secondary structure elements) has fingerprints in the WAXS (wide-angle X-ray scattering) region (Figure 3).

Figure 3

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Schematic illustration of the X-ray solution scattering profile of a typical globular protein (central panel, in magenta) and qualitative signal changes (in green) as a result of (**a**) protein aggregation; (**b**) protein expansion; (**c**) protein tertiary structural changes; (**d**) protein unfolding. In the case of tertiary structural changes, expected difference signals in the WAXS region are of the order of $\sim$1% and are thus better visualized as scattering difference patterns (ΔI versus q). The unfolding of globular proteins can be qualitatively assessed using a Kratky plot ($q^2 I$ versus q). In the case of globular proteins, the Kratky plot presents a peak at $q = \sqrt{3}/R_g$, where R_g is the protein radius of gyration.

as short as 100 ps. For lower time-resolutions, a train of 100 ps X-ray pulses rather than a single pulse can be used to probe the sample, thus increasing the S/N.

X-ray free-electron lasers (XFELs) are an exciting new alternative if sub-picosecond time-resolution is needed. XFELs are able to produce extremely short ($\sim$10 fs) X-ray pulses with a number of photons $\sim 10^2$–10^3 times higher than the $\sim$100 ps pulses produced at synchrotrons. Moreover, XFEL pulses can be focused down to $\sim$0.1 μm without significant reduction in the number of X-ray photons delivered to the sample. XFELs have been used to perform the first steady-state serial femtosecond crystallography (SFX) experiments on protein crystals [3,4•]. In SFX a suspension of nano-to-micro crystals is delivered, usually via a liquid jet, into the path of the X-ray beam. Single diffraction patterns are recorded by a fast pixel array detector whenever an X-ray/crystal hit occurs and these patterns are then assembled into a complete dataset. Similar experiments have been shown to be feasible also at synchrotrons [5–8]. The main advantage of XFELs for dynamic studies is thus that they allow us to address scientific questions that require sub-ps time-resolution.

Several options for time-resolved data collection strategies exist. All are based on the well established classical pump-probe method, where the reaction initiation (pump) is followed by an X-ray probe (or probe series) after a time-delay. In the simplest case of a reversible system which relaxes back to its initial state in a reasonable time (within a few seconds) and without appreciable radiation damage buildup, this can be repeated many times to increase S/N, explore different time-delays and, for crystallography, all needed crystal orientations. In practice, even for readily reversible biological systems, such as naturally photoactive proteins, radiation damage is a significant problem, necessitating either a constant delivery of fresh sample or the dilution of radiation damage over a large sample volume for both scattering and diffraction experiments. An elegant modification for diffraction experiments is the Ratio method where a probe-pump-probe sequence is used and the ratio between the pre and post pump X-ray intensities [9,10], or between independent reference and post pump intensities [11], is determined for each reflection. A recently proposed alternative approach to classic pump-probe experiments is to use Hadamard transform based X-ray probe pulse sequences to improve the time-resolution of experiments using slow detectors or flux limited X-ray sources [12•].

Reaction initiation

There are two requirements that must be considered for reaction initiation. First, the process must be triggered in a significant fraction of the sample and second, the triggering event must be faster than the process of interest.

Rapid mixing

Classical stopped-flow and continuous-flow apparatus are available at several synchrotron beamlines dedicated to time-resolved X-ray scattering experiments,[*] and can be used to study reactions triggered by the rapid mixing of different solutions in the ms [13,14] or even μs time-scales if a continuous-flow apparatus with T-shaped micromixers [15] or jet-in-jet devices are employed [16,17]. In the case of protein crystals, the simplest approach to reaction initiation is to allow the substrate to diffuse into the crystal, where for micro or nano-crystals diffusion times are on the order of ms and μs respectively (Figure 4) [18].

Direct and indirect light activation

To achieve higher time-resolutions reactions must be triggered with a short laser light pulse. This can be used to either directly photoexcite a light sensitive reaction

Figure 4
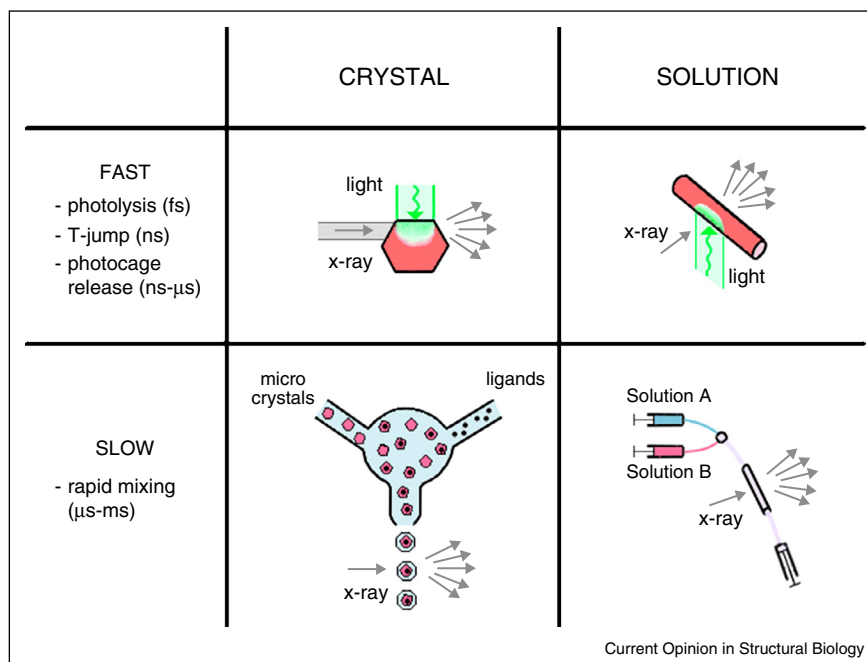
Techniques for triggering protein conformational changes either in crystalline or solution samples. Light activation: light pulses are used to photolyze a bond, rapidly increase the temperature of the solvent surrounding the protein, or to induce the release of a photocaged compound. Rapid mixing: a solution containing either protein crystals or solubilized protein can be mixed with a solution containing a substrate or a denaturant agent and then probed with X-rays to monitor any structural change occurring as a function of time.

[19] or to create a temperature jump [20] that initiates, for example, a folding or enzymatic reaction.

The simplest biological processes to investigate with time-resolved methods are those occurring in inherently light sensitive proteins, many of which can be activated efficiently with short (fs–ns) laser pulses. Unsurprisingly the majority of sub-ms time-resolved structural experiments to date have focused on such systems.

For non-naturally photoactivatable systems, either photolabile protecting groups can be chemically added to the natural substrate, or photocaged unnatural amino acids can be site-specifically incorporated into the protein itself [21•]. However, this is non-trivial. Site-specific labelling of proteins is dependent on the reactivity and accessibility of the residues or requires genetic incorporation of the unnatural amino-acid (with only a few photocaged unnatural amino acids currently available), and the photocaging of substrates can be synthetically challenging [22]. Furthermore, the correct choice of photocaging scaffold is essential as this determines the time-scales accessible.

In all light activated reactions (whether natural or not) the major limitation of photoinitiation is the laser penetration through the sample (Figure 4). Again a balance must be struck between a high sample concentration, desirable to provide good S/N (and unavoidable in a crystallographic experiment), and the need for the laser to uniformly excite the sample volume to be probed.

Data analysis

The result of an X-ray time-resolved experiment, either on a crystalline or solution sample, is usually a set of average multi-dimensional data (integrated Bragg peaks, scattering curves, etc.) at different time-delays from the reaction triggering event. The signal evolution at time-scales longer than few tens of ps, can usually be attributed to exchanges in the population of a finite set of different molecular species each with a well defined (not time-dependent) structure. In such cases, singular value decomposition (SVD) or global analysis (GA) are usually used to extract a limited number of 'components' describing the time-evolution of the experimental signals [23–26]. When a plausible kinetic model is available, it can be used to analyze the data in terms of a generalized GA approach[#] that retrieves the fingerprint of a given physical species on the basis of its predicted time-evolution [11,24,27]. It must be noted that at ultrafast

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[#] While SVD or GA analysis are implemented in several commercial or freely available scientific software packages, a custom fitting routine that matches the kinetic model chosen to describe the observed kinetic changes is required in the case of generalized GA.

time-scales (from fs to ps) the very concept of population kinetics is of course meaningless and every single time point has to be treated as an independent component. The signal time-evolution in this time regime is typically due to structural relaxations within a given minimum of the system free-energy landscape. This corresponds to the observation of a protein within a given energy state dynamically changing its structure as opposed to the observation of an energy-activated transition between two different energy states.

Once the time-independent components have been found, the analysis usually proceeds very differently for crystallography and solution scattering. In both cases, several software packages able to perform standard data analysis have been developed and are freely distributed. Crystallographic data are analyzed in the same way as static data, starting with a known structure to phase the experimentally determined intensities, followed by examination of difference electron density maps to identify the structural changes that occur over time. In the case of solution scattering data, the basis patterns are usually analyzed in terms of global parameters (molecular mass, radius of gyration) or by *ab initio* methods [28]. However, the attribution of experimental time-resolved signals to specific biomolecular motions is usually difficult. This is only partly due to the intrinsic low spatial resolution of solution scattering data. Indeed, although it is conceptually impossible to retrieve a high resolution (all-atom) model of a biomolecule conformation using only its solution X-ray scattering pattern, the combination of TR-SAXS and TR-WAXS data with other detailed structural information either from complementary spectroscopic techniques or from computational approaches can greatly facilitate the structural interpretation of the data [29,30,31•].

Examples/case studies

Time-resolved X-ray crystallography

Time-resolved crystallographic studies allow the structural changes associated with bond breakage/formation, isomerization, side-chain rotation etc. to be visualized across the whole protein, this information when combined with spectroscopic data can provide compelling evidence for the mechanisms of enzyme catalysis and protein function. The dynamic motions that accompany chemical mechanisms, can be used to explain how the structure of the entire protein contributes to both the lowering of the activation energy required for enzyme catalysis and the structural rearrangements that enable signalling and energy transfer. Incomplete photoactivation and mixtures of states produced by parallel reaction pathways can introduce uncertainty during data analysis, by performing time-resolved experiments at multiple temperatures it is sometimes possible to separate the overlapping energy profiles of parallel processes to determine intermediate structures, energy barriers and kinetics.

The photoisomerization and subsequent structural rearrangement of photoactive yellow protein (PYP) has been extensively studied using synchrotron based time-resolved crystallography (most recently [11,32]). Discrepancies between density functional theory calculations and the mechanism deduced from the analysis of time-resolved data have since been highlighted [33,34]. It may be possible to address these discrepancies with ultra-fast experiments using an FEL. Indeed, recent time-resolved SFX experiments on PYP have shown that high-resolution difference electron density maps show improved detail when compared to those obtained from Laue diffraction data [35••]. This has enabled the analysis of the relative occupancies of two intermediate states (pR_1 and pR_2) at 1 μs. Serial time-resolved crystallography has also been applied to photosystem I (PSI) [36] and photosystem II (PSII) [37,38]. For PSI, changes in diffraction were observed at 5 and 10 μs following photoexcitation matching the time-scale of electron transfer from PSI to ferredoxin. The FEL data also allowed the observation of changes in the oxygen evolving complex in PSII indicating binding of a second water molecule during the photocycle.

Time-resolved X-ray solution scattering

A straightforward application of time-resolved X-ray scattering is the study of large conformational changes. These can be monitored by changes in the macromolecule radius of gyration (R_g) from the analysis of the SAXS scattering profile at very small angles in combination with rapid mixing techniques in which a protein solution is combined for example with a substrate solution or a denaturant one [14,15,39].

Time-resolved scattering has also been used in protein aggregation studies. The formation of protein aggregates is naturally and easily detectable in the SAXS region of the scattering pattern [40–42]. However, the large sample heterogeneity (distribution of molecular species with different sizes simultaneously present in solution) can limit the data analysis and makes the data interpretation challenging.

Smaller, faster structural changes, such as allosteric motions or tertiary local changes in protein conformation can also be very effectively studied using time-resolved X-ray scattering [27,43–44,45•,46]. We will illustrate this using the prototypical model system for allosteric proteins, human hemoglobin (Hb). TR-WAXS, in combination with laser photolysis, was used to study the kinetics of the R → T transition in solution [24,47]. The changes in the relative position of the Hb subunits associated with the R → T transition are evident in the WAXS region of the X-ray scattering patterns and the time-resolved data can be accurately described in terms of linear combinations of the deoxyHb (T-state) WAXS pattern and a HbCO (R-state) WAXS pattern [24,48]. Interestingly,

the rate of the Hb allosteric transition was shown to be of the order of ~ 1 μs, that is much faster than previously assumed on the basis of time-resolved optical spectroscopic data [49]. Moreover, in spite of the high heterogeneity that characterizes a cell suspension, it was possible to observe the Hb allosteric transition within intact red blood cells thus demonstrating the feasibility of in-cell TR-WAXS experiments [50].

The advent of XFELs has opened the possibility of investigating the structural dynamics of proteins with fs resolution [51,52]. The extension of time-resolved X-ray scattering techniques to the use of XFELs is straightforward, as demonstrated by a TR-WAXS study on the *Blastochloris viridis* photosynthetic reaction center [53•]. A subpicosecond motion of the reaction center, reminiscent of the so-called 'protein-quake' proposed by Frauenfelder and coworkers for myoglobin (Mb) already 30 years ago [54] was observed, although under conditions of extensive multiphoton absorption by the protein chromophores [53•]. More recent experiments on carbonmonoxy Mb [55•] showed without any ambiguity that protein helices are indeed able to move in the ps time-scale. The strain released by the heme chromophore after mild laser photoexcitation conditions is transferred to the Mb polypeptide chain within a few ps and triggers a damped oscillation of the entire protein. Although synchrotron TR-WAXS experiments clearly indicated that structural changes were occurring within 100 ps [27], their characterization has only been possible with XFELs.

Summary

With the recent developments in X-ray sources (micro-focus beamlines and FELs), high throughput sample delivery methods and data analysis protocols time-resolved X-ray crystallography and solution scattering are becoming increasingly accessible to the wide community interested in investigating biologically relevant macromolecular conformational changes. However, the major obstacle that remains is the challenge of reaction triggering in systems whose mechanisms are not naturally photoinduced. This will only be overcome by bringing together the expertise of X-ray and instrumentation method developers with that of chemists and biologists to develop new and interesting methods of initiation for research questions that will benefit from time-resolved data.

Acknowledgements

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the WAXS and SAXS regions, direct evidence of myoglobin proteinquake
has been obtained. Myoglobin structure was also shown to undergo low
frequency damped oscillations in the picosecond time-scale.