



HAL
open science

Tracking the structural dynamics of proteins with time-resolved X-ray solution scattering

Kevin Pounot, Giorgio Schirò, Matteo Levantino

► **To cite this version:**

Kevin Pounot, Giorgio Schirò, Matteo Levantino. Tracking the structural dynamics of proteins with time-resolved X-ray solution scattering. *Current Opinion in Structural Biology*, 2023, 82, pp.102661. 10.1016/j.sbi.2023.102661 . hal-04213310

HAL Id: hal-04213310

<https://hal.science/hal-04213310>

Submitted on 5 Feb 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

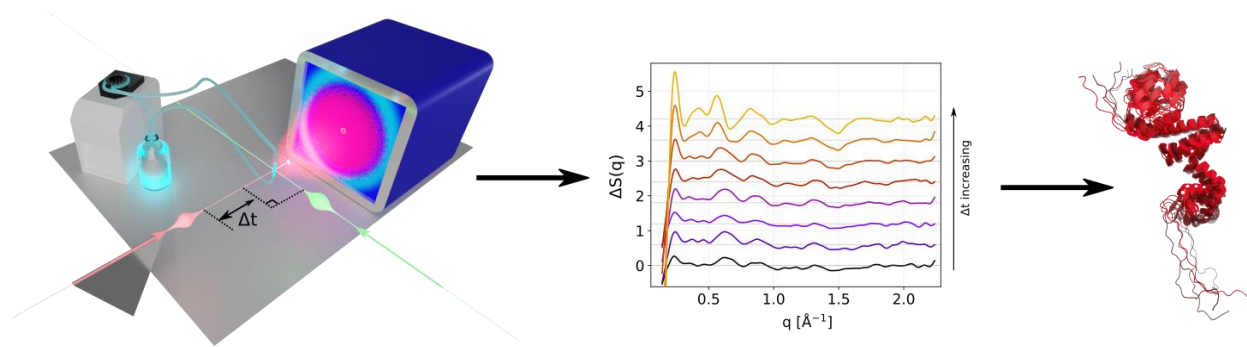
L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Tracking the structural dynamics of proteins with time-resolved X-ray solution scattering

Kevin Pounot¹, Giorgio Schirò², Matteo Levantino¹

¹ ESRF - The European Synchrotron, 71 Avenue des Martyrs, 38043 Grenoble Cedex 9, France

² Univ. Grenoble Alpes, CNRS, CEA, IBS, F-38000 Grenoble, France



Keywords

protein function and motion, biased MD simulations, TR-SAXS/WAXS, synchrotron, XFEL

Abstract

Relevant events during protein function like ligand binding/release, interaction with substrates or with light are often accompanied by out-of-equilibrium structural dynamics. Time-resolved experimental techniques have been developed to follow protein structural changes as they happen in real time after a given reaction triggering event. Time-resolved X-ray solution scattering (TR-XSS) is a promising approach that bears structural sensitivity with temporal resolution in the femto-to-millisecond time range depending on the X-ray source characteristics and the triggering method. Here we present the basic principles of the technique together with a description of the most relevant results recently published and a discussion on the computational methods currently developed to achieve a structural interpretation of the TR-XSS experimental data.

Preprint of: K. Pounot, G. Schiro & M. Levantino, *Curr. Opin. Struct. Biol.*, 82, 102661 (2023)

<https://doi.org/10.1016/j.sbi.2023.102661>

Introduction

Most experimental techniques that are directly sensitive to the structure of biological molecules require either to crystallize or to cryogenically cool the sample, thus trapping each macromolecule in a given state within a solid or at best highly viscous environment. Time-resolved X-ray solution scattering (TR-XSS) was developed in order to probe intramolecular and intermolecular motions of biological molecules in environments where large-scale conformational changes can freely happen [1-4].

Unlike X-ray macromolecular crystallography (MX) or cryoelectron microscopy (Cryo-EM), X-ray solution scattering methods cannot directly yield atomic resolution information due to the averaging effect on the measured signal arising from the orientational distribution of different macromolecules in solution. In spite of this fundamental limitation, TR-XSS has been successfully applied to a large variety of biological systems providing crucial information both on the kinetics and on structural characteristics of functionally relevant motions, as recently reviewed by Ihee and coworkers [5].

While time-resolved spectroscopic techniques typically provide information about local structural changes around a given (intrinsic or extrinsic) chromophore, TR-XSS does not require any protein labeling and all atoms in the biomolecule contribute to the total scattering signal. It is particularly sensitive to global conformation changes but it can also be used to track relatively localized structural changes [6]. In this sense, it is complementary to experimental techniques such as time-resolved optical absorption or Raman spectroscopy, fluorescence resonance energy transfer (FRET) spectroscopy, pulsed electron paramagnetic resonance (pulsed EPR) or nuclear magnetic resonance (NMR).

Another advantage of TR-XSS is that it is sensitive, not only to the structure of macromolecules in solution, but also to that of the surrounding solvent. As the solvent around macromolecules is crucial in determining their function [7], being able to track structural changes of both the solute and the solvent in the same experiment opens new possibilities. Indeed, TR-XSS has been effectively used to monitor changes in the solvent density induced by the energy release from photoexcited proteins to the nearby solvent molecules. This allows correlating protein structural changes with the local temperature increase of the solvent in a TR-XSS experiment [8].

In the following, we will briefly review the most relevant TR-XSS studies that have been published in the last 5 years and highlight the main directions in which experts in the field are moving towards. We will devote particular attention to the studies and approaches that bear the highest potential of having significant impact on biologically relevant systems: 1) TR-XSS on natively photosensitive proteins; 2) the use of photocaged compounds in non photosensitive systems; 3) temperature jumps to trigger protein folding/unfolding. We will not discuss other approaches like rapid mixing as very few successful experiments have been performed so far [9]. Rapid-mixing experiments are more challenging as they cannot be performed in a classical pump-probe fashion and thus are more sensitive to drifts in the background scattering. The final section is an overview of the currently available computational methods for the interpretation of TR-XSS data, which is a critical point for the further development and wider applicability of TR-XSS to a larger number of systems. Indeed, as it is not possible to directly obtain the structure of a protein from an X-ray solution scattering pattern, molecular dynamics (MD) simulations can be used to generate putative model structures that are then refined against the experimental TR-XSS data.

Details about the TR-XSS method have been recently reviewed by Anfinrud and coworkers [10]. Typical samples have a protein concentration of the order of tens of mg/ml and a few hundreds of microliters are typically required to collect transient signals at several time-delays in the

microsecond-to-millisecond (or wider) timescale [10]. Samples are often flown through an X-ray capillary in order to minimize any radiation damage effect from either the X-ray or laser beam [4]. We invite the readers to refer to refs. 4, 5, and 10 (and references therein) for further details on how to set up a TR-XSS experiment. For a detailed description of methods to perform a kinetic analysis of reduced TR-XSS datasets for extracting information on kinetic rate constants and structural intermediates, the reader can refer to the work by Ihee and coworkers [13]. We will limit here to schematically present an example of experimental setup (**Figure 1A**) and a typical reduced dataset (**Figure 1B**). It is important to realize that, starting from *a priori* knowledge of a given system, expected TR-XSS signal can be calculated. This is useful also to get an idea of what kind of structural changes can be detected with this technique. In **Figure 2A** we show a laser induced protein conformational change reported for a bacteriophytochrome [13]. Modifications of the overall structure of bacteriophytochrome (**Figure 2A**) lead to subtle but measurable changes in the absolute scattering pattern (**Figure 2B**). What is measured in TR-XSS are however not absolute scattering patterns directly comparable to the curves reported in Figure 2B, but rather scattering differences (**Figure 2C**). The main challenge in the structural interpretation of TR-XSS data is to make a structural assignment in terms of atomic models able to reproduce the shape of signals like the ones in **Figure 2C**.

Recent applications of TR-XSS

Light activation of intrinsically photosensitive proteins

The possibility of triggering photochemical reactions in solution with light is used to investigate the structural dynamics of proteins naturally sensitive to light. Photon absorption by the light sensitive region of the protein (chromophore) triggers a sequence of functional reactions often accompanied by structural changes that can then be probed by TR-XSS. Time-resolved spectroscopy is often used as a complementary technique to determine the kinetics of spectral changes at the chromophore level, which is important for both designing the TR-XSS experiments and interpreting the kinetics extracted from TR-XSS data.

The phytochrome family, which consists of bilin-containing photoreceptors, has attracted in the last decade the attention of several groups working with TR-XSS [16]. Phytochromes regulate a number of red and far-red light signaling responses in plants and microorganisms and some species are particularly interesting for their potential optogenetic applications [17]. From a structural point of view, phytochromes consist of a globular N-terminal photosensory module and a C-terminal output regulatory module connected together through a flexible hinge region. The photoisomerization of the bilin chromophore is known to induce rearrangements at the photosensory module level. However, the role of the output domain in the full-length phytochrome is still debated [16]. Choudry et al. used TR-XSS together with optical and infrared (IR) spectroscopy to characterize the complex photocycle of an algal phytochrome (DTen Phy) from picoseconds to seconds [18]. TR-XSS results, in particular, clarified that the structural perturbation at the chromophore level, occurring in the picosecond regime, is transmitted to the protein conformation only in the millisecond timescale, when the photoactive state builds up through two intermediate states. Heyes et al. compared the light-induced structural dynamics of a full-length phytochrome from *Synechocystis cyanobacteria* (Cph1) and its truncated form not containing the output domain [19]. The results of this study revealed that the TR-XSS signal is the same in both forms of the protein up to several milliseconds, thus indicating that the signal transduction to the regulatory domain occurs only in the millisecond timescale. Lee et al. used TR-XSS and MD simulations to study the photosensory module of the

bacterial phytochrome BphP from *Pseudomonas aeruginosa* [20]. Comparison of the experimental TR-XSS signal with that calculated from simulated structures allowed the authors to suggest a structural pathway after light absorption in the micro-to-millisecond timescale.

Other TR-XSS studies on different photosensitive proteins have been published in recent years. Westenhoff and coworkers used TR-XSS data compared with atomic models produced by MD simulations to propose the structural rearrangements occurring in phototropin from *Chlamydomonas reinhardtii* [21]. The authors found that the photochemical reaction triggers a rearrangement of the hydrogen bond network along the protein chain from the chromophore to the surface, which in turn induces a movement of functionally relevant protein domains. Ihee and coworkers made use of TR-XSS to investigate the structural changes of a circular photoactive yellow protein oligomer controlled by light [22]. This study elucidated the effect of salt bridges on the kinetic pathway of the allosteric transition in the K30D mutant of homodimeric hemoglobin, where the interfacial salt bridges present in the wild type protein are removed [23].

TR-XSS can be also used to obtain information on ultrafast structural rearrangements around a given protein chromophore. As originally proposed by Frauenfelder and coworkers for myoglobin, a protein reaction is similar to an earthquake: after photolysis of the bond between the ligand and the protein, strain energy is released and a structural deformation propagates from the active site through the protein [24]. This model was termed “proteinquake” and was experimentally confirmed in the case of myoglobin using TR-XSS at an X-ray free-electron laser (XFEL) [25]. More recently, Ihee and coworkers have used the same approach and investigated the ultrafast response of wild type homomeric hemoglobin upon carbon monoxide photodissociation [26] confirming the previous observation of a proteinquake in myoglobin [25]. Moreover, by using MD based structural refinement, the authors managed to identify the most relevant helix motions induced by the proteinquake propagation.

Andreeva et al. succeeded in applying TR-XSS on a system characterized by an extremely low quantum yield (< 1%) to reveal the timescale of the structural changes accompanying the photoactivation of the orange carotenoid protein. Their data suggest that a “numbed” state is formed upon light absorption; this state remains non-photoexcitable for at least 0.5 μ s [27]. Finally, Neutze and coworkers recently proposed a strategy based on MD simulations to explicitly include the X-ray scattering cross term between a membrane protein and the surrounding detergent micelle to model TR-XSS data from solubilized bacteriorhodopsin after light excitation [28]. This work provides a theoretical framework that can significantly aid the interpretation of data from future TR-XSS studies on integral membrane proteins.

Photocaged compounds

Only a small fraction of proteins is naturally photoactive. To overcome this limitation and investigate large-scale conformational changes of proteins that are natively triggered by the interaction with ligands, ions or other substrates, a possible approach is the use of photocaged compounds [29,30]. In such compounds, the protein ligand is bound to a small organic molecule through a covalent, but photolabile bond. A light pulse at appropriate wavelength can be used to quickly cleave (photolyze) this bond, thus releasing the free ligand in solution and making it readily available for protein binding.

Photocaged adenosine triphosphate (ATP) in combination with TR-XSS has been recently used to investigate functionally relevant biological systems [31-33].

After the proof-of-principle study by Josts et al. [31], who used laser-induced release of ATP to track the dimerization of MsbA nucleotide binding domains, Andersson and co-workers have recently studied the structural dynamics of the ATP-dependent calcium pump protein SERCA [32]. Upon pumping calcium ions from the cytoplasm to the sarcoplasmic reticulum, SERCA undergoes major structural changes involving several intermediates. While most of these intermediates had already been characterized with static X-ray crystallography [34], Ravishankar et al. were able to identify for the first time a long-lived structural intermediate that allows ADP release after phosphorylation using TR-XSS in combination with MD simulations [32] (see further info in the section on simulation methods).

A further study from the Andersson group used again photocaged-ATP to investigate the open-to-close transition of adenylate kinase (AdK) [33]. AdK is an enzyme with an ATP-binding domain and a domain able to bind adenosine monophosphate (AMP-binding domain). The binding of one ATP and one AMP molecule triggers a conformational change that brings the two domains closer to each other, and allows the catalysis of the phosphoryl transfer reaction that ultimately produces two adenosine diphosphate (ADP) molecules. Orädd et al. used TR-XSS to observe a transient intermediate state showing a partial closing of the ATP and AMP domains and a partial unfolding of the ATP-binding domain, which causes the breaking of an arginine-aspartate salt bridge. The structural interpretation is based on comparison with models generated through equilibrium MD simulations and it is supported by independent equilibrium NMR measurements on an AdK mutant lacking the arginine-aspartate salt bridge [33].

The above studies were performed using the commercially available 1-(2-nitrophenyl)-caged ATP (NPE-ATP). The main limitation of this compound is that its decaging time constant, which is pH dependent, is of the order of a few millisecond (4.5 ms at pH 7) [35]. Still ATP hydrolysis is at the base of a large number of biological reactions, thus we foresee that the same approach will be applied to many other systems that undergo relevant conformational changes in the millisecond-to-seconds (or longer) timescale.

Protein Folding/Unfolding

The mechanistic details accompanying the transition from the active folded state to the inactive unfolded state (and vice versa) of a protein has been largely investigated but is still poorly characterized from a structural point of view. TR-XSS offers a new approach to monitor in real-time the structural changes that occur during protein folding/unfolding. Indeed, by combining TR-XSS with solvent heating by an IR laser pulse, it is possible to monitor the effect of an “instantaneous” (compared to the time scale of protein folding/unfolding) temperature increase (T-jump) on both the solvent and protein structure [8,36-39]. Experiments are typically performed by exciting water vibrational modes in the near-IR with a nanosecond laser and probing the induced structural changes with TR-XSS. By comparing the TR-XSS signal in the water scattering region ($\sim 2 \text{ \AA}^{-1}$) with steady-state XSS data of water (or buffer) measured at different temperatures, it is possible to estimate the T-jump induced in the protein solution [8].

Fraser and coworkers investigated T-jump induced conformational changes of Cyclophilin A (CypA), a proline isomerase that facilitates protein folding and moderates intracellular signaling pathways [36]. The authors were able to resolve two distinct relaxations: 1) a first relaxation occurring within a few microseconds from the T-jump and attributed to the increased dynamics of a surface loop adjacent to the protein active site; 2) a second relaxation occurring within tens of microseconds and interpreted as a small contraction of the protein core of CypA. Interestingly, the latter relaxation is not observed in a protein mutant (S99T) where the catalytic activity is impaired due to the absence

of a key rotameric exchange on the active site. This suggests that the conformational change corresponding to the second relaxation is necessary to trigger the enzyme catalytic activity. T-jump TR-XSS measurements on wild-type CypA at different starting temperatures (in the 5-30 °C range) allowed the authors to also estimate the activation enthalpy and entropy of the two kinetic steps. Being able to estimate not only equilibrium enthalpies and entropies, but also activation ones is crucial in order to obtain a detailed picture of the energy landscape of a protein and thus of protein function. For this reason, it is worth noting that TR-XSS can help retrieving this kind of information also in the case of sub-millisecond reactions.

Another interesting example was reported by Lin Chen and coworkers on α -lactalbumin, a 123-residue protein with a well-defined molten globule state [38]. The analysis of their TR-XSS data, collected from 20 μ s to 70 ms from a T-jump, revealed three different intermediates (I, U_1 and U_2). Unfolding can proceed either by first populating the I intermediate and then the U_1 state ($F \rightarrow I \rightarrow U_1$), or directly by going from the folded state to the U_2 intermediate ($F \rightarrow U_2$). The I intermediate is a molten globule state with a similar topology to the native folded state, but with less condensed secondary structures. The U_1 and U_2 intermediates are two partially unfolded states characterized by higher disorder and large protein radius of gyration (R_g).

Simulation methods for TR-XSS data interpretation

TR-XSS data results from an average over many orientations and conformations, which implies that there is no direct way to obtain an atomic detailed structure from the scattering differences. Although the structural sensitivity is enhanced by extending the data collection up to the wide-angle X-ray scattering (WAXS) region, the information content of solution scattering patterns is not enough to avoid the risk of over-interpretation if randomly generated structural models are used as candidates for structural refinement. To mitigate this problem, MD simulations are valuable tools as they add physico-chemical information and provide reasonable conformational ensembles from which X-ray scattering patterns can be accurately computed and compared with experimental data [40]. Several softwares like CRY SOL [41], FoXS [42] or WAXSiS [15] are able to calculate the X-ray solution scattering pattern of a protein provided that an atomic model is available [42].

The interpretation of the experimental TR-XSS data in terms of well-defined protein structural changes has been so far the main bottleneck of the technique. Indeed, while TR-XSS is able to detect even relatively small structural changes and allows to accurately measure the kinetics of these changes, it is not possible to obtain detailed information on the atomic displacements directly from the data. The use of MD simulation methods is the most promising approach to overcome this bottleneck as demonstrated by several examples in the recent literature.

The MD simulations make use of optimized force fields initially designed to reproduce equilibrium structures but multiple methods have since then been developed to explore also out-of-equilibrium, on-pathway structures [44] (**Figure 3**). A common strategy to this end is to introduce an additional energy term to the force field, a so-called *bias*, which is used to drive the simulation towards a desired state starting from the available experimental information on the system. When the bias is an energy penalty associated with the distance between the simulated structure and a reference one, the simulation is commonly referred to as *targeted MD*. A similar approach consists in introducing an energy penalty based on the distance between calculated and experimental X-ray scattering patterns pairs. We refer to the latter biasing method as *XSS guided MD*.

The first structural refinements of intermediate states tracked with TR-XSS have used standard equilibrium MD without any bias energy term [45]. For each frame in the simulation, a scattering pattern was computed and a scoring function was used to select the structures matching the experimental data. More recently, the strategy to select relevant sub-ensembles has been improved by implementing clustering methods such as genetic algorithms [21,22,33,36,46]. Especially when calculated structures are compared with data collected in the sub-nanosecond timescale, it is important to include in the MD simulation the effect of the excess energy deposited on the chromophore after laser photoexcitation. This can be done by locally increasing the kinetic energy of the chromophore at the beginning of the simulation [47, 48]

Another important direction is to take into account the solvent contribution to the scattering patterns. Indeed, popular programs for the calculation of XSS patterns, like CRY SOL [41] or FoXS [42], estimate such contribution “implicitly” by treating the solvent (and solvation shell layer) as a *continuum*, which limits the accuracy of the results especially at high scattering angles [43]. The possibility to include the solvent atoms in the calculation of the scattering patterns was first proposed and successfully used by Merzel and Smith [49]. Hub and coworkers later used explicit-solvent calculations in XSS-guided structure refinement to reproduce experimental results of several different proteins [50]. The same method has been used since then by other authors [38,50,51,52]. A recent example is the work of Hsu et al. [51], where the transition from a random-coil to a α -helix deca-alanine peptide could be simulated by taking the hydration layer contribution explicitly into account.

Unbiased MD simulations, although have yielded successful results, require long computing time on dedicated supercomputers to reach timescales often observed in TR-XSS experiments (microseconds or longer). Introducing a bias helps in accelerating the exploration of otherwise inaccessible states. When crystal structures are available, intermediate states can be found using *targeted MD* [32,38,51]. A recent example involves the membrane protein SERCA mentioned above [32]. Here the simulation approach is complicated by the need of modeling the contribution of lipids to the overall scattering signal. Ravishankar et al. initially performed several targeted MD simulations in the absence of membrane lipids. In all of them, the starting structure was that of the fully opened Ca^{2+} bound state and the final one was one of different existing crystallographic structures of SERCA intermediate species. Already at this step, it was possible to isolate models yielding a better agreement with TR-XSS data than the crystallographic ones. After addition of the lipids to these models, further equilibrium MD simulations were performed. The authors were able to obtain a slightly better agreement with TR-XSS data by including the effect of the surrounding lipids. They found out that most simulated structures were very similar to each other, which *a posteriori* demonstrates the robustness of an approach that has allowed them to unveil for the first time a crucial structural intermediate in the SERCA structural transition.

In cases where the crystal structure of the initial and/or final state is not at hand, but some prior knowledge on the reaction and the associated structural transition is available, it is possible to apply more localized biases such as a pulling force on definite atoms. Lee et al. have applied such bias to either two residues or to the center-of-mass of different domains of *Pseudomonas aeruginosa* bacteriophytochrome (PaBphP) [20]. Thanks to this approach, the authors were able to demonstrate that PaBphP undergoes first a slight opening of the α -helices in the photosensory domain and later a more dramatic structural change that was never observed in other bacteriophytochromes. The simulations were also useful to rule out an alternative possible structural change that had been proposed to happen after photoexcitation.

The aforementioned methods make use of the experimental TR-XSS data only indirectly. A more direct way is to use TR-XSS guided MD to bias the simulation. This can be achieved by modifying standard packages for MD simulations like GROMACS [54] or NAMD [55] and has been done by

several authors [38,50,51,56]. As an example, the TR-XSS data from the T-jump experiment on bovine α -lactalbumin described above [38] have been interpreted using TR-XSS guided MD simulations. Thanks to this approach, refined structures in good agreement with both steady-state small-angle X-ray scattering and circular dichroism measurements (both in terms of the α -helix content and of the estimated R_g) were obtained. We foresee that TR-XSS guided MD will further develop and become more widely used in the next future.

All the above studies suggest that the field is becoming mature enough to develop standard easy-to-use tools that will allow a wider community to extract relevant structural information on intermediates of a given protein functional transition. Other approaches able to better describe non-equilibrium states (e.g. by integrating QM/MM methods) may still be needed for particularly demanding systems.

Conclusion

The TR-XSS method, applied to biological systems, initially earned its stripes with intrinsically photosensitive systems [1, 2]. The method has continuously improved over the years and recently it has been successfully coupled with new approaches to trigger protein conformational changes such as T-jump or the use of photocaged compounds.

The signal obtained in TR-XSS experiments is a low-resolution, one-dimensional picture of an averaged protein structure. Hence, only indirect methods allow an atomic-detailed interpretation of the scattering difference signal. In recent years, the use of MD simulations has become the method of choice for structural interpretation of TR-XSS data. Performing MD simulations remains difficult for the non-expert user as it has a steep learning curve, but progress is being continuously made towards more user-friendly tools.

As illustrated in the present review, several works published in the last 5 years made use of TR-XSS to investigate a large variety of important biological systems. The most relevant advancements have been the use of new triggering methods and of MD simulations for TR-XSS data interpretation (**Figure 4**). TR-XSS is the tool of choice for detecting global structural changes of a protein in solution and the formation of structural intermediates with very high temporal resolution. The availability of user-friendly tools to run TR-XSS guided MD simulations can revolutionize the field and open up the use of this kind of technique to the vast structural biology community.

Acknowledgments

We would like to thank Dr. Marco Cammarata for many insightful discussions over the years and for being a continuous inspiration for our research activity. This work was supported by the French national research agency (ANR) through grant ANR-21-CE11-0036 ("PhotoGene").

Figure 1

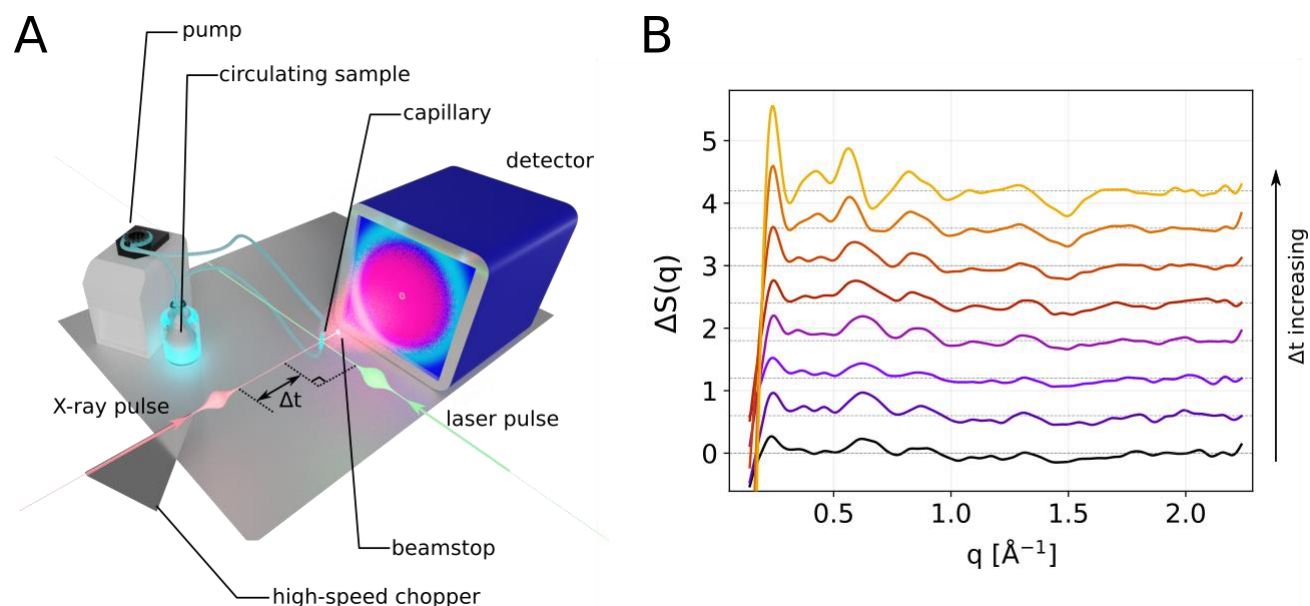


Figure 1 - Time-resolved X-ray solution scattering (TR-XSS). (A) Schematic representation of a typical experimental setup: a short X-ray pulse (in red) is used to probe the sample at different time delays with respect to an excitation laser pulse (in green). At the ID09 beamline of the ESRF, a high-speed chopper is used to isolate either a single 100 ps X-ray pulse or a train of pulses (overall duration of a few microseconds) depending on the storage ring filling mode [11, 12]. The sample can be sealed in a capillary or, as sketched in the figure, circulated through the capillary by means of a pump. A beamstop is placed as close as possible to the sample to minimize the contribution of air scattering from the primary beam. (B) Typical time-resolved difference patterns from a protein in solution. The signal is obtained by subtracting the “laser off” scattering pattern (scattering measured without the laser or before the laser hits the sample) from the scattering measured at a time delay Δt with respect to the arrival of the laser pulse. The resulting curves contain the contribution of both the protein structural changes and the solvent changes induced by the release of energy from the protein to the solvent (heating). The solvent heating contribution can be measured in a separate TR-XSS experiment and is typically removed from the difference curves ΔS to highlight the protein contribution [1]. The zero difference level is indicated for each difference curve by the dotted gray line. The data reported in panel B are relative to a TR-XSS experiment performed at the ID09 beamline in which the conformational changes of the chromophore binding domain of CarH from *Thermus thermophilus* were followed after photoexcitation at 532 nm (Pounot et al., unpublished results). These data are shown here only for illustrative purposes and will be the subject of a separate dedicated publication.

Figure 2

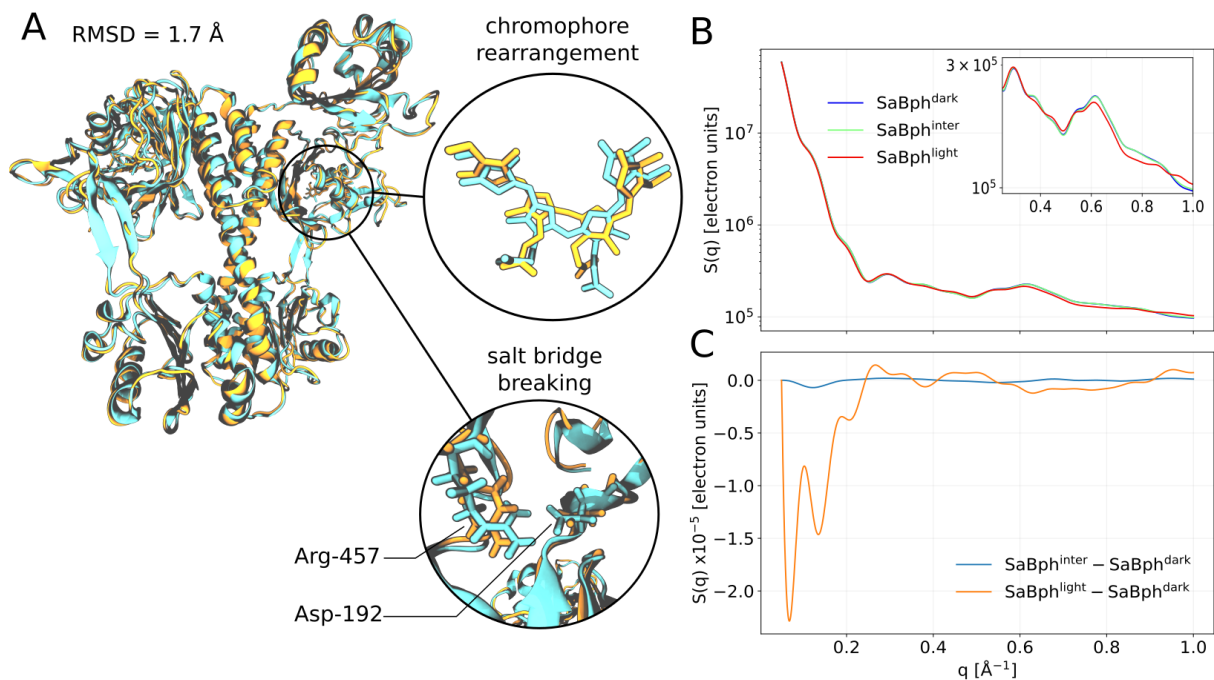


Figure 2 - Effect of protein conformational changes on difference scattering patterns. (A) The structure of bacteriophytochrome from *Stigmatella aurantiaca* (SaBph) in the dark form (SaBph^{dark}, PDB 6PTQ, cyan cartoon and licorice representation) and in a 5 ns photo-activated form (SaBph^{light}, PDB 7JR5, orange cartoon and licorice representation) are superimposed for comparison. After 5 ns from photoexcitation the chromophore structure is significantly different from the ground state one; moreover, the relative distance of several helices has changed and a functionally relevant salt bridge is broken [14]. The root mean-square deviation (RMSD) for the two structures is 1.7 Å. **(B and C)** For illustrative purposes, we artificially divided the overall conformational change in an initial step in which only the chromophore rearrangement has occurred (we call this artificial state SaBph^{inter}) and a second step in which also the polypeptide conformational change is present (SaBph^{light}). The absolute scattering patterns corresponding to these three states have been calculated using the WAXSiS webserver [15] and are reported in panel **B**, the inset representing an enlarged view of the high scattering angles region. While the three absolute patterns have the same overall shape, they differ in small details that are directly linked to the structural changes represented in panel **A**. This is better highlighted by plotting the difference between two scattering patterns, which is typically referred to as the TR-XSS signal. **(C)** Comparison of the SaBph^{inter}-SaBph^{dark} scattering difference (blue) with the SaBph^{5ns}-SaBph^{dark} scattering difference (orange). The panel highlights that an overall (although small) protein structural change results in a much larger TR-XSS signal than a structural change localized on the chromophore.

Figure 3

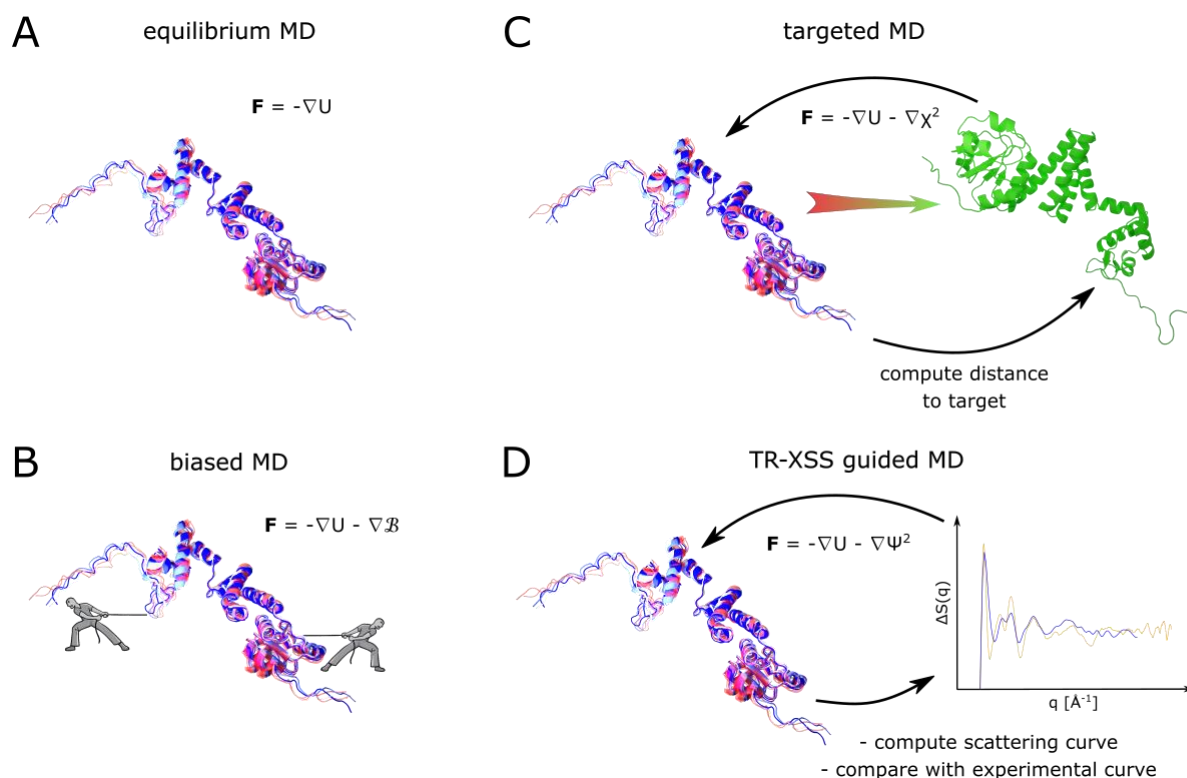


Figure 3 - Simulation methods to interpret TR-XSS data. **(A)** In the case of equilibrium (unrestrained) MD, a starting protein structure changes with time under the action of the force \mathbf{F} applied to each atom. In this case \mathbf{F} is simply calculated as the derivative of the chosen force field (potential energy U) with respect to the atomic positions. In the figure, the time evolution of the protein structural change is represented through 5 different structures evolving from blue to red (with increasing transparency). **(B)** In biased MD, an additional energy term \mathcal{B} is used to avoid getting stuck in local energy minima during the simulation. The bias term can arise from an artificial force on specific atoms or from any other local interaction relevant for the system under study. **(C)** In targeted MD, the atoms are constrained to move towards a target structure, represented in green here, by introducing a metric (χ^2) to evaluate the distance between the starting and the target structure. **(D)** The TR-XSS guided MD acts similarly to the targeted MD, but the metric (ψ^2) measures the distance between the experimental scattering curve (dark yellow line, data is top curve from **Figure 1B**) and that computed using the simulation coordinates (blue line).

Figure 4

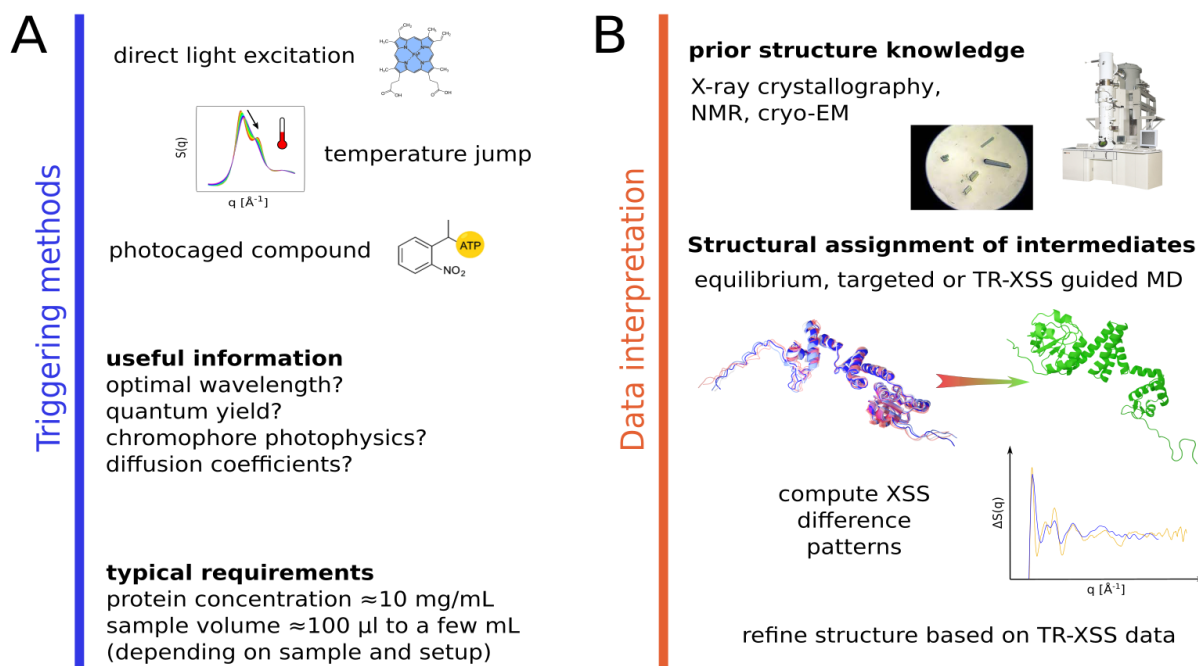


Figure 4 - Overview on triggering methods and data interpretation. (A) Summary of the triggering methods described in the manuscript along with brief information useful when designing and preparing a TR-XSS experiment. **(B)** Workflow for data interpretation of TR-XSS data based on MD simulation methods.

References

1. Cammarata M, Levantino M, Schotte F, Anfinrud PA, Ewald F, Choi J, Cupane A, Wulff M, Ihee H: **Tracking the structural dynamics of proteins in solution using time-resolved wide-angle X-ray scattering.** *Nat Methods* 2008, **5**:881–886.
2. Andersson M, Malmerberg E, Westenhoff S, Katona G, Cammarata M, Wöhri AB, Johansson LC, Ewald F, Eklund M, Wulff M, et al.: **Structural dynamics of light-driven proton pumps.** *Structure* 2009, **17**:1265–1275.
3. Levantino M, Yorke BA, Monteiro DC, Cammarata M, Pearson AR: **Using synchrotrons and XFELs for time-resolved X-ray crystallography and solution scattering experiments on biomolecules.** *Curr Opin Struct Biol* 2015, **35**:41–48.
4. Orädd F, Andersson M: **Tracking Membrane Protein Dynamics in Real Time.** *J Membr Biol* 2021, **254**:51–64.
5. Lee Y, Lee H, Ihee H: **Structural dynamics of proteins explored via time-resolved x-ray liquidography.** *Chem Phys Rev* 2022, **3**:041304.
6. Andersson M, Vincent J, van der Spoel D, Davidsson J, Neutze R: **A proposed time-resolved X-ray scattering approach to track local and global conformational changes in membrane transport proteins.** *Structure* 2008, **16**:21–28.
7. Fenimore PW, Frauenfelder H, McMahon BH, Parak FG: **Slaving: solvent fluctuations dominate protein dynamics and functions.** *Proc Natl Acad Sci U S A* 2002, **99**:16047–16051.
8. Cho HS, Schotte F, Stadnytskyi V, DiChiara A, Henning R, Anfinrud P: **Dynamics of Quaternary Structure Transitions in R-State Carbonmonoxyhemoglobin Unveiled in Time-Resolved X-ray Scattering Patterns Following a Temperature Jump.** *J Phys Chem B* 2018, **122**:11488–11496.
9. Josts I, Gao Y, Monteiro DCF, Niebling S, Nitsche J, Veith K, Gräwert TW, Blanchet CE, Schroer MA, Huse N, et al.: **Structural Kinetics of MsbA Investigated by Stopped-Flow Time-Resolved Small-Angle X-Ray Scattering.** *Structure* 2020, **28**:348–354.e3.
10. Cho HS, Schotte F, Stadnytskyi V, Anfinrud P: **Time-resolved X-ray scattering studies of proteins.** *Curr Opin Struct Biol* 2021, **70**:99–107.
11. Wulff M, Plech A, Eybert L, Randler R, Schotte F, Anfinrud P: **The realization of sub-nanosecond pump and probe experiments at the ESRF.** *European Synchrotron Radiation Facility. Faraday Discuss* 2003, **122**:13–26.
12. Cammarata M, Eybert L, Ewald F, Reichenbach W, Wulff M, Anfinrud P, Schotte F, Plech A, Kong Q, Lorenc M, et al.: **Chopper system for time resolved experiments with synchrotron radiation.** *Rev Sci Instrum* 2009, **80**:015101.
13. Oang KY, Yang C, Muniyappan S, Kim J, Ihee H: **SVD-aided pseudo principal-component analysis: A new method to speed up and improve determination of the optimum kinetic model from time-resolved data.** *Struct Dyn* 2017, **4**:044013.
14. Carrillo M, Pandey S, Sanchez J, Noda M, Poudyal I, Aldama L, Malla TN, Claesson E, Wahlgren WY, Feliz D, et al.: **High-resolution crystal structures of transient intermediates in the phytochrome photocycle.** *Structure* 2021, **29**:743–754.e4.
15. Knight CJ, Hub JS: **WAXSiS: a web server for the calculation of SAXS/WAXS curves based on explicit-solvent molecular dynamics.** *Nucleic Acids Res* 2015, **43**:W225–30.

16. Takala H, Björling A, Berntsson O, Lehtivuori H, Niebling S, Hoernke M, Kosheleva I, Henning R, Menzel A, Ihalainen JA, et al.: **Signal amplification and transduction in phytochrome photosensors.** *Nature* 2014, **509**:245–248.
17. Chernov KG, Redchuk TA, Omelina ES, Verkhusha VV: **Near-Infrared Fluorescent Proteins, Biosensors, and Optogenetic Tools Engineered from Phytochromes.** *Chem Rev* 2017, **117**:6423–6446.
18. Choudry U, Heyes DJ, Hardman SJO, Sakuma M, Sazanovich IV, Woodhouse J, De La Mora E, Pedersen MN, Wulff M, Weik M, et al.: **Photochemical Mechanism of an Atypical Algal Phytochrome.** *Chembiochem* 2018, **19**:1036–1043.
19. Heyes DJ, Hardman SJO, Pedersen MN, Woodhouse J, De La Mora E, Wulff M, Weik M, Cammarata M, Scrutton NS, Schirò G: **Light-induced structural changes in a full-length cyanobacterial phytochrome probed by time-resolved X-ray scattering.** *Commun Biol* 2019, **2**:1.
20. Lee SJ, Kim TW, Kim JG, Yang C, Yun SR, Kim C, Ren Z, Kumarapperuma I, Kuk J, Moffat K, et al.: **Light-induced protein structural dynamics in bacteriophytochrome revealed by time-resolved x-ray solution scattering.** *Sci Adv* 2022, **8**:eabm6278.
- * The authors identified two intermediates from a kinetic analysis of TR-XSS during the photoconversion of the bacteriophytochrome from *Pseudomonas aeruginosa*. They made use of biased MD simulations to generate structural models for the intermediates and have highlighted important motions of the alpha-helices that occur upon photoexcitation.
21. Henry L, Berntsson O, Panman MR, Cellini A, Hughes AJ, Kosheleva I, Henning R, Westenhoff S: **New Light on the Mechanism of Phototransduction in Phototropin.** *Biochemistry* 2020, **59**:3206–3215.
22. Lee SJ, Kim Y, Kim TW, Yang C, Thamilselvan K, Jeong H, Hyun J, Ihee H: **Reversible molecular motional switch based on circular photoactive protein oligomers exhibits unexpected photo-induced contraction.** *Cell Rep Phys Sci* 2021, **2**:100512.
23. Choi M, Kim JG, Muniyappan S, Kim H, Kim TW, Lee Y, Lee SJ, Kim SO, Ihee H: **Effect of the abolition of intersubunit salt bridges on allosteric protein structural dynamics.** *Chem Sci* 2021, **12**:8207–8217.
24. Ansari A, Berendzen J, Bowne SF, Frauenfelder H, Iben IE, Sauke TB, Shyamsunder E, Young RD: **Protein states and proteinquakes.** *Proc Natl Acad Sci U S A* 1985, **82**:5000–5004.
25. Levantino M, Schirò G, Lemke HT, Cottone G, Glowina JM, Zhu D, Chollet M, Ihee H, Cupane A, Cammarata M: **Ultrafast myoglobin structural dynamics observed with an X-ray free-electron laser.** *Nat Commun* 2015, **6**:6772.
26. Lee Y, Kim JG, Lee SJ, Muniyappan S, Kim TW, Ki H, Kim H, Jo J, Yun SR, Lee H, et al.: **Ultrafast coherent motion and helix rearrangement of homodimeric hemoglobin visualized with femtosecond X-ray solution scattering.** *Nat Commun* 2021, **12**:3677.
- * XFEL based TR-XSS with femtosecond resolution was used to investigate the ultrafast response of wild type homomeric hemoglobin upon carbon monoxide photodissociation. The work confirmed previous observations obtained on the proteinquake in myoglobin and proposed a simulation based structural assignment of the intermediates states forming in the sub-picosecond to picosecond time regime.
27. Andreeva EA, Niziński S, Wilson A, Levantino M, De Zitter E, Munro R, Muzzopappa F, Thureau A, Zala N, Burdzinski G, et al.: **Oligomerization processes limit photoactivation and recovery of the orange carotenoid protein.** *Biophys J* 2022, **121**:2849–2872.
- * TR-XSS was used to investigate the photoactivation of the orange carotenoid protein. The formation of a numbered (non-photoexcitable) transient state was observed for the first time.

From a methodological point of view, it is remarkable that a structural change from a low quantum yield (< 1%) system could be detected with TR-XSS using a multiple laser pulse excitation scheme.

28. Sarabi D, Ostojić L, Bosman R, Vallejos A, Linse J-B, Wulff M, Levantino M, Neutze R: **Modeling difference x-ray scattering observations from an integral membrane protein within a detergent micelle.** *Struct Dyn* 2022, **9**:054102.
 - * Paper introducing a theoretical framework to describe the contribution of the detergent micelle that is typically used in TR-XSS membrane protein studies. The authors used MD simulated structures to evaluate the contribution of X-ray scattering cross terms between solubilized bacteriorhodopsin and the surrounding detergent micelle.
29. Monteiro DCF, Amoah E, Rogers C, Pearson AR: **Using photocaging for fast time-resolved structural biology studies.** *Acta Crystallogr D Struct Biol* 2021, **77**:1218–1232.
30. Klán P, Šolomek T, Bochet CG, Blanc A, Givens R, Rubina M, Popik V, Kostikov A, Wirz J: **Photoremovable protecting groups in chemistry and biology: reaction mechanisms and efficacy.** *Chem Rev* 2013, **113**:119–191.
31. Josts I, Niebling S, Gao Y, Levantino M, Tidow H, Monteiro D: **Photocage-initiated time-resolved solution X-ray scattering investigation of protein dimerization.** *IUCrJ* 2018, **5**:667–672.
32. Ravishankar H, Pedersen MN, Eklund M, Sitsel A, Li C, Duelli A, Levantino M, Wulff M, Barth A, Olesen C, et al.: **Tracking Ca ATPase intermediates in real time by x-ray solution scattering.** *Sci Adv* 2020, **6**:eaaz0981.
 - ** Intermediate state that plays a crucial role in the membrane protein SERCA identified for the first time by combining TR-XSS and targeted MD simulations. Reaction triggering obtained by laser induced ATP release from a photocaged compound.
33. Orädd F, Ravishankar H, Goodman J, Rogne P, Backman L, Duelli A, Nors Pedersen M, Levantino M, Wulff M, Wolf-Watz M, et al.: **Tracking the ATP-binding response in adenylate kinase in real time.** *Sci Adv* 2021, **7**:eabi5514.
 - ** TR-XSS was used to track a transient state during catalytic activity of the enzyme adenylate kinase. The structural characterization of this transient state was obtained by combining MD simulations in a membrane environment with TR-XSS measurements. They obtained a detailed picture of the conformational changes that occur during the release of ADP.
34. Dyla M, Kjærgaard M, Poulsen H, Nissen P: **Structure and Mechanism of P-Type ATPase Ion Pumps.** *Annu Rev Biochem* 2020, **89**:583–603.
35. McCray JA, Herbette L, Kihara T, Trentham DR: **A new approach to time-resolved studies of ATP-requiring biological systems; laser flash photolysis of caged ATP.** *Proc Natl Acad Sci U S A* 1980, **77**:7237–7241.
36. Thompson MC, Barad BA, Wolff AM, Sun Cho H, Schotte F, Schwarz DMC, Anfinrud P, Fraser JS: **Temperature-jump solution X-ray scattering reveals distinct motions in a dynamic enzyme.** *Nat Chem* 2019, **11**:1058–1066.
37. Henry L, Panman MR, Isaksson L, Claesson E, Kosheleva I, Henning R, Westenhoff S, Bernthsson O: **Real-time tracking of protein unfolding with time-resolved x-ray solution scattering.** *Struct Dyn* 2020, **7**:054702.
38. Hsu DJ, Leshchev D, Kosheleva I, Kohlstedt KL, Chen LX: **Unfolding bovine α -lactalbumin with T-jump: Characterizing disordered intermediates via time-resolved x-ray solution scattering and molecular dynamics simulations.** *J Chem Phys* 2021, **154**:105101.
 - ** The authors combined T-jump TR-XSS with equilibrium MD and TR-XSS guided MD to reveal

two unfolding pathways for α -lactalbumin. The MD simulations provided information about the radius of gyration and α -helices content for each ensemble corresponding to the intermediates.

39. Chan AM, Nijhawan AK, Hsu DJ, Leshchev D, Rimmerman D, Kosheleva I, Kohlstedt KL, Chen LX: **The Role of Transient Intermediate Structures in the Unfolding of the Trp-Cage Fast-Folding Protein: Generating Ensembles from Time-Resolved X-ray Solution Scattering with Genetic Algorithms.** *J Phys Chem Lett* 2023, **14**:1133–1139.
40. Bernetti M, Bussi G: **Comparing state-of-the-art approaches to back-calculate SAXS spectra from atomistic molecular dynamics simulations.** *The European Physical Journal B* 2021, **94**:180.
41. Svergun D, Barberato C, Koch MHJ: **CRY SOL– a Program to Evaluate X-ray Solution Scattering of Biological Macromolecules from Atomic Coordinates.** *Journal of Applied Crystallography* 1995, **28**:768–773.
42. Schneidman-Duhovny D, Hammel M, Tainer JA, Sali A: **FoXS, FoXSDock and MultiFoXS: Single-state and multi-state structural modeling of proteins and their complexes based on SAXS profiles.** *Nucleic Acids Res* 2016, **44**:W424–9.
43. Cupane A, Levantino M: **Investigating protein structure and dynamics through wide-angle X-ray solution scattering.** *Il Nuovo Cimento C* 2016, **39**:303.
44. Hénin J, Lelièvre T, Shirts MR, Valsson O, Delemotte L: **Enhanced Sampling Methods for Molecular Dynamics Simulations.** *Living J Comp Mol Sci* 2022, **4**:1583.
45. Malmerberg E, Omran Z, Hub JS, Li X, Katona G, Westenhoff S, Johansson LC, Andersson M, Cammarata M, Wulff M, et al.: **Time-Resolved WAXS Reveals Accelerated Conformational Changes in Iodoretinal-Substituted Proteorhodopsin.** *Biophys J* 2011, **101**:1345–1353.
46. Kim TW, Lee SJ, Jo J, Kim JG, Ki H, Kim CW, Cho KH, Choi J, Lee JH, Wulff M, et al.: **Protein folding from heterogeneous unfolded state revealed by time-resolved X-ray solution scattering.** *Proc Natl Acad Sci U S A* 2020, **117**:14996–15005.
47. Arnlund D, Johansson LC, Wickstrand C, Barty A, Williams GJ, Malmerberg E, Davidsson J, Milathianaki D, DePonte DP, Shoeman RL, et al.: **Visualizing a protein quake with time-resolved X-ray scattering at a free-electron laser.** *Nat Methods* 2014, **11**:923–926.
48. Brinkmann LUL, Hub JS: **Ultrafast anisotropic protein quake propagation after CO photodissociation in myoglobin.** *Proc Natl Acad Sci U S A* 2016, **113**:10565–10570.
49. Merzel F, Smith JC: **SASSIM: a method for calculating small-angle X-ray and neutron scattering and the associated molecular envelope from explicit-atom models of solvated proteins.** *Acta Crystallogr D Biol Crystallogr* 2002, **58**:242–249.
50. Chen P-C, Hub JS: **Validating solution ensembles from molecular dynamics simulation by wide-angle X-ray scattering data.** *Biophys J* 2014, **107**:435–447.
51. Hsu DJ, Leshchev D, Kosheleva I, Kohlstedt KL, Chen LX: **Integrating solvation shell structure in experimentally driven molecular dynamics using x-ray solution scattering data.** *J Chem Phys* 2020, **152**:204115.
52. Nijhawan AK, Chan AM, Hsu DJ, Chen LX, Kohlstedt KL: **Resolving Dynamics in the Ensemble: Finding Paths through Intermediate States and Disordered Protein Structures.** *J Phys Chem B* 2021, **125**:12401–12412.
53. Schlitter J, Engels M, Krüger P: **Targeted molecular dynamics: a new approach for searching pathways of conformational transitions.** *J Mol Graph* 1994, **12**:84–89.
54. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJC: **GROMACS: fast, flexible, and free.** *J Comput Chem* 2005, **26**:1701–1718.

55. Phillips JC, Hardy DJ, Maia JDC, Stone JE, Ribeiro JV, Bernardi RC, Buch R, Fiorin G, Hénin J, Jiang W, et al.: **Scalable molecular dynamics on CPU and GPU architectures with NAMD.** *J Chem Phys* 2020, 153:044130.
56. Björling A, Niebling S, Marcellini M, van der Spoel D, Westenhoff S: **Deciphering solution scattering data with experimentally guided molecular dynamics simulations.** *J Chem Theory Comput* 2015, 11:780–787.