A guide to time-resolved structural analysis of light-activated proteins

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Introduction

Dynamical changes in protein structures are essential for protein function and occur over femtoseconds to seconds timescales. X-ray free electron lasers have facilitated investigations of structural dynamics in proteins with unprecedented temporal and spatial resolution. Light-activated proteins are attractive targets for time-resolved structural studies, as the reaction chemistry and associated protein structural changes can be triggered by short laser pulses. Proteins with different light-absorbing centres have evolved to detect light and harness photon energy to bring about downstream chemical and biological output responses. Following light absorption, rapid chemical/small-scale structural changes are typically localised around the chromophore. These localised changes are followed by larger structural changes propagated throughout the photoreceptor/photocatalyst that enables the desired chemical and/or biological output response. Time-resolved serial femtosecond crystallography (SFX) and solution scattering techniques enable direct visualisation of early chemical change in light-activated proteins on timescales previously inaccessible, whereas scattering gives access to slower timescales associated with more global structural change. Here, we review how advances in time-resolved SFX and solution scattering techniques have uncovered mechanisms of photochemistry and its coupling to output responses. We also provide a prospective on how these time-resolved structural approaches might impact on other photoreceptors/photoenzymes that have not yet been studied by these methods.

Abbreviations
AdoCbl, adenosylcobalamin; BLUF, blue light using Flavin; bR, bacteriorhodopsin; FAD, flavin adenine dinucleotide; FAP, fatty acid photodecarboxylase; GAF, cGMP phosphodiesterase/adenyl cyclase/FhlA; HBI, hydroxybenzylidene imidazolinone; LOV, light-oxygen-voltage; OEC, oxygen-evolving complex; PAS, Per/Arnt/Sim; pCA, p-coumaric acid; PHY, phytochrome-specific; POR, protochlorophyllide oxidoreductase; PYP, photoactive yellow protein; SFX, serial femtosecond crystallography; SSX, serial synchrotron crystallography; TR-SAXS, time-resolved small-angle X-ray scattering; TR-WAXS, time-resolved wide-angle X-ray scattering; XFEL, X-ray free electron laser.

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peak brightness of XFEL light pulses are many orders of magnitude higher, allowing smaller sample size, and they have a uniquely short pulse length in the order of femtoseconds [3]. These characteristics have enabled the development of serial femtosecond crystallography (SFX) [4,5] and time-resolved SFX (TR-SFX) [6], in addition to solution scattering techniques to visualise structural and chemical changes. In combination, these techniques can follow protein structural change across the femtoseconds to seconds (fs-s) timescale. These state-of-the-art dynamic structural biology approaches are therefore ideally positioned to interrogate the mechanisms of light-activated proteins (e.g. photoreceptors, photosynthetic proteins and photoenzymes) by triggering the natural photochemistry using very short laser pulses and following chemical and structural change across multiple timescales. Here, we highlight recent insights of mechanism gained using SFX, time-resolved X-ray scattering and allied biophysical approaches, to study chemical and structural change at the molecular level on previously inaccessible timescales, and a prospective on other light-activated systems, likely to be amenable to similar studies in the future. In synergy with time-resolved spectroscopy and computational methods, time-resolved structural studies will provide the much-needed insight into photochemistry, signal transduction mechanisms (photoreceptors) and catalysis (photoenzymes) that underpin the mode of action of photoreceptor proteins and photo-biocatalysts in biology.

Most living organisms have evolved to detect and respond to light. This ability is vital for a myriad of crucial processes, including photosynthesis [7], vision [8,9] and circadian biology [10,11]. Cellular systems have also evolved to mitigate the harmful effects of photooxidative damage [12-14]. Known light-activated proteins and their salient features are summarised in Table 1 and these depend on chromophores to detect light in the visible region (380-760 nm wavelength range) of the electromagnetic spectrum (Fig. 1) [15]. Owing to their unique ability to respond to light, photo-activated proteins have found unique and important applications in the fields of optogenetics and synthetic biology [16-19] and super-resolution light microscopy [20,21]. A number of desirable properties, including reversibility, noninvasive nature of activation and control, genetic ‘encode-ability’ and excellent spatio-temporal regulation of the photochemical response, make light-activated proteins attractive targets in a range of applications [22]. For example, fluorescent proteins, which can respond to different wavelengths of light, have been used as exogenous probes in imaging where they have revolutionised the field of cell biology [23,24].

The photocycle of a light-activated protein is often defined as the sequence of light-induced transitions through a range of transient intermediate states. The absorption of a photon by the chromophore in the resting dark state triggers a reaction cascade that occurs over a wide range of timescales from femtoseconds through to seconds, characterised by changes in the structure of the chromophore and the protein itself (Fig. 2, a typical example of a photocycle). These intrinsic structural/chemical dynamics that lead from one structure to the next are crucial to overall function. Time-resolved spectroscopic techniques have been used extensively to characterise different intermediates and chemical steps in photocycles [25]. However, commonly used electronic spectroscopic methods are sensitive only to changes in the light detecting chromophore and its immediate environment and do not report on more global structural change that dictates the output response. Direct visualisation of these chemical and structural changes, across relevant timescales, is now possible due to advances in SFX and X-ray scattering methods [3].

Scene-setting: a brief overview of time-resolved serial femtosecond crystallography and scattering techniques

X-ray free electron lasers are classified as fourth-generation X-ray sources and have transformed the field of structural biology. XFELs generate X-ray pulses approximately three to four orders of magnitude shorter (10-100 fs) than a typical shortest pulse from a traditional synchrotron source (100 ps). Furthermore, the short duration of the XFEL pulse does not compromise intensity, as a typical pulse (shorter than 30 fs) contains a similar number of photons as a 1 s synchrotron exposure [26]. While such exposure conditions lead to sample destruction, XFEL light sources rely on the diffraction-before-destruction principle. Indeed, Neutze et al. [27] predicted that, under XFEL exposure conditions, sample disintegration due to radiation damage is slower than diffraction, providing the possibility to determine damage-free macromolecular structures [28]. However, as a crystal only yields a single diffraction image before disintegration, a large number of crystals in random orientations are needed to obtain a complete dataset/crystal structure [29]. Consequently, in a typical experiment thousands of diffraction images are collected from individual crystals in a serial manner at room temperature, which
involves presenting crystals to the pulsed X-ray beam (Fig. 3A). Moreover, as the requirement to cryo-cool crystals is removed in TR-SFX measurements, the likelihood of triggering a reaction \textit{in crystallo} is also increased at room temperature.

Conformational changes along the reaction pathway of light-activated proteins can be followed with exquisite spatial and temporal resolution by TR-SFX conducted according to a pump-probe scheme [6]. A reaction is triggered in a crystal (pump) at \( t = 0 \) and subsequent diffraction data (probe) are collected after a defined period of time \( \Delta t \). This sequence is repeated to collect a complete diffraction data set, from which a structural model is determined that represents a snapshot of the light-induced changes having taken place at time delay \( \Delta t_1 \) (Fig. 3B). Molecular movies can then be generated in an approach where the same process is repeated for several pump-probe delays \( \Delta t_1, \Delta t_2, \ldots, \Delta t_j \), leading to individual time-resolved structures (i.e. movie frames) that represent the conformational changes in response to illumination over time. In principle, complete structural characterisation of the photocycle of a light-activated biological system can be carried out using this approach.

Motions of a protein can differ in the crystalline and in the solution states [30] and macromolecular
processes are generally slowed down in the crystal due to the presence of viscous crystallisation agents and packing contacts [31]. The latter can in rare cases induce subtle structural constraints that halt enzyme catalysis at an intermediate state [32]. Conversely, a recent XFEL study to understand large conformational changes in crystallo by using crystals of adenine riboswitch aptamer RNA (riboA) showed synchronous ligand-induced molecular rearrangements were possible, causing no disruption to the lattice order [33]. However, often, large-scale conformational changes, such as extensive domain motions, may not be fully allowed in the crystal so that it is important to also interrogate dynamics in solution, where the full motional range can be probed (see as an example the discussion on the photoactive yellow protein (PYP) below [34]). Time-resolved solution scattering is one such approach (Fig. 3A). Time-resolved small-angle X-ray scattering (TR-SAXS) and time-resolved wide-angle X-ray scattering (TR-WAXS) therefore complement time-resolved crystallography methods, in providing a more complete understanding of dynamics following light activation [35]. Time-resolved SAXS yields information on the size and shape of the protein at low resolutions [36,37], whereas time-resolved WAXS is used to probe more localised changes at higher resolutions [38,39]. The former allows detection of changes in the radius of gyration (Rg) of a protein in response to light excitation, whereas the latter is sufficiently sensitive to probe spatial perturbations of an individual protein subunit, domain or even secondary structure element [40]. A pump-probe scheme can also be used with S/WAXS at both synchrotron [41] and XFEL [42,43] sources.

Different methods can be used to induce fast (bio)chemical reactions, including visible [44], IR [45] and
X-ray pulses [46] and electric field stimulation [47]. Light pulses are the most frequently used way to trigger a reaction and study dynamical processes with characteristic timescales that span many decades (fs–ms). Micromixers and other jet-in-jet devices [48] can also be used for rapid mixing [49] of protein and ligand to trigger a reaction but their use does not allow higher temporal resolution (ns–µs) [50]. Light activation has also been possible with proteins that are not naturally light-activated by using photolabile chemicals and ligands or photocaged substrates. For example, the dynamics of myoglobin have been visualised by both synchrotron-based Laue crystallography [51,52] and TR-SFX [44] after triggering cleavage of the photosensitive Fe–CO bond. Notable are helix movements both towards and away from the haem cofactor, and changes in the spatial orientation of the haem pyrrole rings. TR-S/WAXS have been used to visualise local and global structural changes following CO dissociation in both haemoglobin [41] and myoglobin [42]. Similarly, motions in cytochrome c oxidase [53,54] (ps–ns timescales) were found to be associated with chemical change (i.e. Fe–CO bond cleavage) in the enzyme active site. In NO reductase—a complex multicentre, haem-containing enzyme—motions have been observed using a photolabile precursor of NO [55]. Here, the 2.1 Å structure revealed an initial intermediate state characterised by a slight bending of the Fe–N–O coordination geometry, providing a rationale for the stability of the Fe–NO complex in the enzyme active site. The use of non-natural light-activated systems has provided important insights into photochemically induced dynamics. However, the power of time-resolved structural methods becomes even more evident when working with natural light-activated proteins, which through evolution have become optimised to couple photoreception with downstream photocatalysis or chemical/structural signalling, and we discuss these below with prominent case studies that have emerged in recent years.

Dynamics and photosynthesis

Many of the light-activated proteins required for photosynthesis, such as photosystem I and II (PSI and II), bacterial reaction centres and light-harvesting complexes, have become prime targets for time-resolved structural studies. These systems have been extensively

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**Fig. 2.** A typical photocycle of a light-activated protein, PYP showing the different transient intermediates formed. A structural transition at around 0.6 ps in PYP has been associated with trans-to-cis isomerisation of the chromophore pCA as studied by TR-SFX (shown in the inset overlay of dark (yellow, PDB id 5HD3) and 0.8 ps after light illumination (green, PDB id 5HDD) structures [105]. Structural changes during PYP photocycle probed using time-resolved S/WAXS showed a significant elongation along the long axis of the protein in the signalling state (pB₃). The solution structures have been adapted with permission from reference [34]. Copyright (2016) American Chemical Society.
studied and the mechanisms and timescales of electron transfer have been documented in most cases [56,57]. However, uncovering structural changes associated with charge separation/electron transfer has been more challenging, in the main due to the large nature of the multisubunit complexes involved. Early proof-of-principle studies using TR-SFX illustrated how XFEL sources can be harnessed to probe the dynamics of photosynthetic proteins using crystals of PS I in complex with the soluble electron transfer protein ferredoxin [58]. Visible light was used to trigger electron transfer between PS I and ferredoxin, and diffraction probed at 5 and 10 μs delays. A detailed analysis of the structural rearrangements was not possible due to incomplete diffraction data obtained for each time delay. However, the differences in the observed diffraction intensities compared to the ground (dark) state identified structural changes linked to electron transfer and subsequent dissociation of the PS I-ferredoxin complex. The time range over which these changes were observed agrees with previously published kinetic data [59,60], thereby demonstrating the utility and relevance of time-resolved structural methods in studies of light-activated systems.

PS II remains by far the most extensively studied by TR-SFX. It is a multisubunit membrane–protein complex that catalyses light-driven water oxidation at the Mn4CaO5 oxygen-evolving complex (OEC) [61,62]. The reversible photocycle of PS II was proposed to comprise four sequential photon absorption steps, generating four intermediate redox states. These different redox states (S0–S4) are formed during the photocycle.
on the μs-ms timescales [63] but could not be characterised using traditional crystallography. Furthermore, radiation damage caused by X-ray photons meant that the Mn atoms in the OEC were reduced, which resulted in inconsistent geometries of the cluster [64]. The mechanism of water oxidation and O=O bond formation, which are key to understanding PS II function and ultimately, all life on Earth, has therefore remained elusive. The development of SFX and TR-SFX techniques may finally allow this to be addressed, together with other important questions about the mechanism of PS II, such as the dynamics of electron transfer, localisation of essential subunits and the evolution of oxygenic photosynthesis [56,61]. Comparison of high-resolution crystal structures obtained using traditional synchrotron [65] and XFEL [66] sources illustrated the advantage of XFEL ‘damage-free’ diffraction data, with compact geometries reported for the radiation-sensitive OEC. Notably, the Mn-Mn distance in the OEC was found to be shorter by 0.1–0.2 Å in a XFEL-determined 1.95 Å structure of the S1 redox state. Several other initial TR-SFX studies failed to characterise the different intermediate states due to the relatively small magnitude of the structural changes, which were difficult to observe at the resolutions achieved (between 4.5 to 5.2 Å) [67–69]. A 2.25 Å room temperature structure of the S3 state-reported small perturbations (~ 0.1 Å) in metal distances in the OEC [70]. Moreover, a 2.35 Å structure of the S3 state showed displacement of a water molecule and simultaneous appearance of a new oxygen atom in the OEC [71] (Fig. 4A). Although further improvements in resolution are necessary, it is clear that SFX has the potential to ultimately provide dynamic structural information for the elusive water splitting mechanism during photosynthesis [72–75].

Recently, ultrafast structural changes on ps timescales in a photosynthetic reaction centre were studied using TR-SFX [76]. These results show the structural perturbations in the pair of chlorophyll molecules and the recurring movements of Cα backbone atoms, which accompany the primary charge-separation event in photosynthetic reaction centre in response to light absorption. In addition to TR-SFX experiments, time-resolved solution scattering experiments have also been carried out to study protein quake-like motions in the photosynthetic reaction centre of the bacterium Blastochnorhis viridis [43]. Following excitation, proteins undergo quake-like rapid structural motions, releasing strain as structural changes propagate outwards from an epicentre [77,78]. Time-resolved WAXS measurements, collected using XFEL radiation, identified ultrafast global conformational changes in the photosynthetic reaction centre following excitation, involving recurring movements in the Cα atoms of the transmembrane domains on the picosecond timescale. These snapshots have provided an understanding of how structural deformations release strain acquired after energy absorption by the cofactors in the photosynthetic reaction centre. Even if the biological relevance

Fig. 4. Use of TR-SFX to study dynamics in photosystem II and rsEGFP2. (A) Overlay of dark (PDB id 5WS5) and illuminated (PDB id 5WS6) structures showing small structural changes in the locations of atoms comprising the OEC of Photosystem II [71]. Insertion of water (oxygen) is indicated by dashed arrows. Location of the Ca, Mn and O atoms of the dark structure is shown as spheres in darker shades of green, violet and red, respectively. (B) Overlay of the 10 ns rsEGFP2 intermediate-state (cyan, PDB id 6T3A) and off-state (violet, PDB id 6T39) structures depicting the chromophore HBI and the surrounding regions [81]. The figure shows the presence of the cis isomer after light excitation and the resulting rearrangement of the residues in the vicinity. (C) Chromophore twisting in rsEGFP2 observed 1 ps after light activation. The off state structure is shown in grey (PDB id 5O8A) and the twisted intermediate in red (PDB id 5O8C). Chromophore twisting at 1 ps is accompanied by a shift in the entire helix that carries the chromophore [84].
of the results reported in [43] is affected by the multiphoton excitation conditions used, in principle protein quakes may be functionally important in other energy-transducing proteins, such as PS I and II, light-harvesting complexes and bacterial rhodopsin or sensory receptors such as PYP and phytochromes. Time-resolved scattering techniques are perhaps the most effective tools to study such phenomena, which have also been demonstrated in other non-natural light-activated protein systems [41,44,51]. An XFEL-based time-resolved X-ray scattering study on myoglobin [42] used excitation conditions more biologically relevant (~ 1.5 photons per chromophore) to show that the perturbation triggered by light absorption at the active site level propagates to the global protein structure at the acoustic speed of sound, thus confirming the protein quake hypothesis. The time evolution of global structural parameters (radius of gyration and volume) revealed that protein atoms collectively oscillate during the quake, thus highlighting the relevance of underdamped low-frequency vibrations in proteins.

**Photoswitchable fluorescent proteins**

The photoswitchable fluorescent protein rsEGFP2 has many applications in nanoscale imaging of live cells [79]. It has a hydroxybenzylidene imidazolinone (HBI) chromophore that undergoes a reversible cis-trans isomerisation and changes in protonation state between the fluorescent on-state (absorbing at 479 nm) and the nonfluorescent off-state (absorbing at 403 nm). Whereas cryo-crystallography provided a static view on the on- and off-state structures [80], structural changes occurring during photoswitching remained elusive, as well as the order in which isomerisation and chromophore (de)protonation occur. Motivated by answering these open questions, TR-SFX experiments have been carried out and captured an intermediate formed 10 ns after photoexcitation of rsEGFP2 during off-to-on transitions of the chromophore [81]. The intermediate structure demonstrated that the trans-cis transition of the protonated chromophore had already taken place at this time point. The phenolic group of the chromophore was displaced ~ 4.5 Å in the cis isomer, a change accompanied by perturbations in the residues lining the rsEGFP2 chromophore-binding pocket (Fig. 4B). Even if transient absorption spectroscopy [81] confirmed an earlier suggestion [82] that the deprotonation of the chromophore occurs as a ground-state process following isomerisation, the sequence and timescale of isomerisation and (de)protonation events remain controversially discussed [83]. When probed at a shorter timescale of 1 ps, the chromophore appeared to be fully twisted representing an excited state intermediate halfway between the trans and cis isomers that has been predicted by QM/MM simulations [84]. A shift of the entire helix carrying the chromophore facilitates this twisting of HBI (Fig. 4C). Furthermore, at 3 ps after illumination, the excited state twisted population decreased in favour of the cis isomer. These new structural insights have since facilitated the engineering of a variant with improved photoswitching characteristics [84]. It has also been shown that cis-trans isomerisation of photoswitchable fluorescent proteins can be influenced by crystal packing since motions of the surrounding β-barrel motif of the protein are involved [85].

**Natural photoreceptors**

Several distinct chromophores (e.g. flavin, bilin, retinal, coenzyme B12) are found in natural biological photoreceptors and a number have been investigated using XFEL sources. In these systems, chromophore photochemistry elicits structural changes that ultimately trigger a biological signalling output. The overall structural dynamics following light excitation of sensor histidine kinases [86] and light–oxygen–voltage (LOV) domains [87,88] of phototropins have been visualised by time-resolved S/WAXS. Structural dynamics in orange carotenoid proteins from cyanobacteria [89] and signal-active rhodopsin-GPCR complex [90] has also been probed using SAXS, albeit not in a time-resolved study. However, most studies have focused on rhodopsins, PYP and the phytochrome superfamily. All of these involve an ultrafast chromophore isomerisation that triggers a cascade of protein structural change, from localised dynamics around the chromophore to slower and more global motions in the protein. We summarise below current knowledge with prominent systems.

**Rhodopsins**

Bacteriorhodopsin (bR) is a retinal containing transmembrane protein involved in the unidirectional pumping of protons from the cytoplasm to the exterior of the cells. Its photocycle has been studied extensively and several crystal structures of intermediates at cryogenic temperatures have been reported [91]. However, there is considerable variation in reported structures, which has been complicated by radiation-induced damage. This has meant that some details of the photochemical mechanism have remained poorly understood. A proof-of-principle study of bR dynamics was conducted at room temperature and resulted in
a 2.3 Å resolution structure of an intermediate using lipidic-cubic phase grown bR crystals [92]. A more extensive TR-SFX study on bR showed that the photoisomerisation of retinal had already taken place prior to the earliest recorded time point at 16 ns (Fig. 5A) [93]. The isomerised retinal adopts a highly distorted conformation and the covalent bond between C15 of the retinal and a conserved Lys residue is twisted. As the chromophore straightens, it displaces a conserved tryptophan residue on the cytoplasmic side of the protein and a water molecule on the extracellular side of the protein. These motions are believed to disperse energy and optimise the isomerisation quantum yield, and in turn trigger a cascade of structural rearrangements and proton pumping [reviewed recently [94]]. Subsequently, isomerisation intermediates in bR were captured by ultrafast TR-SFX studies on the sub-ps timescale [95,96]. These were carried out in the multiphoton excitation regime [97], a caveat common to all ultrafast TR-SFX studies [98]. Ultrafast structural changes in a protein related to bR, a retinal-dependent sodium pump (KR2), have also been studied across multiple (fs–ms) timescales using TR-SFX [99]. Structural snapshots of intermediate stages in the photocycle confirmed that isomerisation of retinal occurs on the fs timescale followed by rearrangements of the chromophore-binding pocket (ns–µs timescales). The high-resolution structures obtained enabled identification of transient sodium binding sites close to retinal and structural insight into the mechanism of unidirectional pumping of ions across the membrane against a concentration gradient. Recently, TR-SFX studies on another retinal-dependent chloride ion-pumping rhodopsin (CIR) were reported on ps timescales [100]. Retinal isomerisation in CIR closely resembles that in KR2, although as Cl− is larger than Na+ or H+ the retinal motion is much more pronounced compared to both KR2 and bR to allow its displacement towards the intracellular direction. TR-SFX data on channelrhodopsin (ChR) from *Chlamydomonas reinhardtii* collected on timescales ranging from µs-ms showed the displacement of isomerised retinal towards the proton donor residues, which results in shift of transmembrane helix TM3 [101]. However, major open

Fig. 5. Time-resolved SFX and scattering studies on different photoreceptors. (A) bR superposition of the Δτ = 16 ns (blue, PDB id 5B6W) and Δτ = 290 ns (purple, PDB ID 5H2J) structures upon the resting state bR structure (grey, PDB id 5B6V) showing the isomerisation of retinal and changes in the neighbouring Trp residue [93]. (B) Shape comparison of the Pr state (pink) and Pfr state (green) of the full-length Cph1 phytochrome. The crystal structure of the photosensory region is superimposed and shown as pink sticks (figure taken from reference [117], Copyright 2019, Springer Nature). (C) A combination of scattering data and crystal structures showed that the phytochrome sensory region consisting of PAS-GAF-PHY domains undergoes a closed (dark, PDB id 400P) to open (illuminated, PDB id 4001) transition upon light illumination. The secondary structure elements in the tongue region of PHY domain are highlighted in magenta [110].

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questions remain in the rhodopsin field—for example, if and how long-range cooperative effects between individual monomers in bR (which is a trimer) influence dynamics.

Photoactive yellow protein

Photoactive yellow protein is a small protein (14 kDa), first found in the photosynthetic bacterium *Halorhodospira halophila*. It is a blue light-detecting sensory photoreceptor involved in phototaxis [102]. The chromophore, *p*-coumaric acid (pCA), absorbs a photon of blue light and undergoes a rapid *trans*-to-*cis* photoisomerisation, which triggers a series of reversible reactions that comprises the photocycle of PYP. These reactions take place over extensive (ps–ms) timescales and involve numerous intermediates, some of which have been characterised using time-resolved Laue crystallography at synchrotron sources [103,104]. TR-SFX has captured the rapid *trans*-to-*cis* isomerisation of pCA after ca 0.6 ps and shown that this isomerisation is accompanied by displacement of some atoms by as much as 1.3 Å (Fig. 2) [105]. Twisting of the *trans* state of pCA begins as early as ~140 fs after excitation and progressively increases until the *cis* state becomes the abundant species after ~590 fs. Further TR-SFX measurements at longer time delays of 10 ns and 1 μs [106] have shown broad agreement with data obtained by Laue diffraction [103,104,107,108].

To study the effects of the *trans*-to-*cis* isomerisation of pCA at a more global structural level, the entire PYP photocycle has been investigated over a wide time range from 100 ps to 1 s by collecting time-resolved S/WAXS scattering patterns [34]. A conformational contraction caused by the photoisomerisation is followed by relaxation along the long axis to produce the PYP signalling state. The radius of gyration (*Rg* = 16.6 Å) of this light state was found to be significantly larger than that for the dark state PYP (*Rg* = 14.7 Å; Fig. 2). This real-time partial unfolding of the protein was impossible to detect in TR-SFX studies due to constraints imposed by the crystal contacts. Recent time-resolved absorption spectroscopy studies showed that the photocycle kinetics and structural dynamics of PYP differ markedly between the crystalline and solution state [30]. Some intermediates observed in the solution state were not identified in the crystalline state and *vice versa* and were attributed to the constraints imposed by the crystal contacts and the lower hydration level in the crystalline state. This underlines again the importance of acquiring data in both solution and crystal states and under the same buffer conditions. In that regard, PYP is an exemplar of how a combination of time-resolved scattering and SFX can provide complete pictures of photochemically induced structural transitions using a combination of approaches.

Phytochromes

The phytochrome superfamily is photosensory proteins found in plants, fungi and bacteria and is normally sensitive to red (Pr state) and far-red (Pfr state) light through their bilin chromophores [109]. The linear tetrapyrrole chromophore is tethered to the photosensing core of the protein and undergoes ultrafast *Z/E* isomerisation at the C15-C16 double bond upon excitation with red light. The photosensing core is comprised of Per/Arnt/Sim (PAS), cGMP phosphodiesterase/adenyl cyclase/FhlA (GAF) and phytochrome-specific (PHY) domains, while the output domains, the site of the signalling response, are generally located at the C terminus and vary between different species. The mechanism by which light-induced electronic excitation of bilin yields the eventual photoresponse was poorly understood prior to implementing time-resolved structural studies. The first time-resolved X-ray scattering studies (μs–ms timescale) of the photosensory region (PAS-GAF-PHY domains) of a bacteriophytochrome [110] indicated large structural changes of the order of nanometers in the PHY domain following illumination. These structural movements were not detected in a truncated protein comprising only the PAS and GAF domains, thus underlining the importance of conformational changes in the PHY domains in signal transduction to the output domains. The crystal structures of both Pr (dark) and Pfr (illuminated) states showed a transition from a closed to an open state following illumination (Fig. 5C). This transition was accompanied by further changes in the tongue region of the PHY domains, which transformed from a β-sheet in the Pr state to α-helix and loop in the Pfr model.

The first TR-SFX data for the chromophore-binding domains of *Deinococcus radiodurans* phytochrome at 1 ps after excitation were recently reported [111]. The 2.21 Å resolution data revealed a twisting of the D-ring of the bilin and the photo-dissociation of a conserved water molecule, which transiently disrupts the interaction between the bilin and the protein. These motions are accompanied by ultrafast backbone and water movements around the bilin, which allow the PHY tongue to alter its conformation and trigger the photoresponse. Further TR-SFX data collected after 5 ns and 33 ms on another bacteriophytochrome from *Stigmatella aurantiaca* has provided important insights into the displacement of the covalently bound bilin...
chromophore [112]. It was suggested that rearrangement of the water network and amino acid residues in the bilin-binding pocket, together with changes at the dimeric interface, aids the propagation of the signal towards the PHY domains.

Additional time-resolved SAXS studies on a number of full-length bacterial phytochromes revealed structural evolution over micro- and millisecond timescales after photoexcitation, leading to a twisting motion of the histidine kinase output domain with respect to the chromophore-binding domains [113–116]. Structural rearrangements on the microsecond timescale are small and restricted to the chromophore-binding domain, whereas the global structural changes in the output domain only occur a few milliseconds after initiation of the photoreaction. These results are important as they provide deeper understanding of the signalling mechanism of full-length bacterial phytochromes.

Other studies have investigated structural changes following photoactivation in a full-length cyanobacterial phytochrome (Cph1) using time-resolved S/WAXS [117]. The structural dynamics in Cph1 were found to be more complex when compared to the full-length bacterial phytochromes, with multiple structural transitions over timescales running into seconds, leading to an opening motion of part of the dimer interface in the output domain (Fig. 5B). Studies with an algal phytochrome also showed similar structural transitions on millisecond timescales, but with differences in the chromophore-binding dynamics on shorter timescales (microseconds) [118]. These illustrate that the structural dynamics vary significantly between different types of phytochrome, and may reflect different levels of complexity in transduction of the light signal to the output domain. TR-SFX with the GAF domain of a related phytochrome-like protein—the cyanobacteriochrome PixJ which alternates between blue (Pb) and green (Pg) light-absorbing states of the bilin cofactor—has been reported [119]. It was shown that PixJ protein crystals are susceptible to X-ray damage by synchrotron radiation. A 1.55 Å resolution model of the dark-adapted Pb state has been determined at room temperature using XFEL radiation. These crystals were able to photoconvert between the two states making PixJ a prime target to discover early structural events associated with phytochrome photochemistry.

Expanding the scope of time-resolved SFX and S/WAXS

The examples illustrated here demonstrate the power of cutting-edge time-resolved SFX and S/WAXS methods to elucidate structural transitions associated with, and induced by, the photochemistry found in natural light-activated systems. Nature has supplied us with many natural light-activated proteins, and the examples discussed above suggest that combined TR-SFX and TR-S/WAXS investigations will be informative. Photo receptor proteins remain prime candidates for XFEL-based research; so far, TR-SFX studies have been limited to chromophores that undergo an isomerisation reaction or to study rapid movements (around chromophores and domains) in photosynthetic proteins triggered by light absorption. There is scope to extend TR-SFX to flavin-based photoreceptors, for example cryptochromes, blue light using Flavin (BLUF) domains [120] or LOV domains and the recently discovered coenzyme B12-dependent photoreceptor CarH, which regulates the expression of carotenoid biosynthetic genes in photosynthetic bacteria [121–124]. CarH exists as a tetramer (dark state) and binds DNA. Upon illumination, B12 photochemistry (Co–C bond photolysis occurring on the ps–ns timescale) triggers major structural change (µs–ms), leading ultimately to tetramer disassembly and gene transcription [125,126] (Fig. 6A). Crystal structures of the dark and light-adapted states of CarH have been reported [127], but structural knowledge of these transitions in solution is currently lacking. The hierarchy in timescales for the fast photochemical transitions and slower global changes is suited to a combination of SFX and S/WAXS approaches.

There are also excellent opportunities to work with a very distinct class of photoactive proteins (i.e. light-activated enzymes). Photo-biocatalysis is an emerging field: recent efforts have focused on the photocatalytic properties of NAD(P)H- and flavin-dependent enzymes [128,129]. There are only a few naturally occurring light-activated enzymes, namely DNA photolyase, fatty acid photodecarboxylase (FAP) and protochlorophyllide oxidoreductase (POR). A photoexcited flavin cofactor is used by DNA photolyase to sense blue light and initiate an electron transfer pathway that leads to catalysis [130,131]. Several structures have been solved to rationalise the photochemistry of DNA repair, but unambiguous detection of intermediates in the reaction photocycle remains a challenge [132]. FAP also utilises a flavin cofactor to catalyse decarboxylation of fatty acids to alkanes using blue light [133]. FAP is an important biocatalyst with numerous biotechnological applications [134,135]. A catalytic cycle has been proposed based on time-resolved spectroscopy measurements and a medium-resolution crystal structure of the ‘resting’ enzyme [133]. Time-resolved spectroscopy studies have started to open up chemical understanding of the reaction mechanism.
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Recently, a high-resolution crystal structure, together with TR-SFX, FTIR, time-resolved vibrational and electronic spectroscopies, and quantum chemical calculations, provided detailed insight into the photocycle of FAP (Fig. 6B) and its spectroscopic and structural intermediate states [138]. Incidentally, the radiation damage-free SFX structure of the resting state of the enzyme revealed an unusual bending of the oxidised flavin chromophore that is suggested to play a role in FAP catalysis [138]. Further TR-SFX experiments are required for more detailed insight into intermediate-state structures and reaction products of the photocatalytic process.

There are also superb opportunities to advance our understanding of photocatalysis with POR—involved in the light-dependent biosynthesis of chlorophyll—especially in the area of enzymatic H-transfer [139] which occurs using a multistep light-driven reduction of the substrate protochlorophyllide (Pchlide) [140,141]. High-resolution crystal structures of the POR–NADPH complexes have been solved from which models of photocatalysis have emerged [142] (Fig. 6C). Photoactive crystals of the POR–NADPH–Pchlide complex remain elusive, but if obtained would open up the exciting prospect of TR-SFX studies of reaction chemistry and associated structural transitions.

**Conclusions and outlook**

Remarkable technological progress over the past decade has facilitated a deeper and more fundamental understanding of several natural light-activated proteins. In all light-responsive proteins studied to date, a hierarchy of timescales for structural transitions starts with ultrafast and localised dynamics in and around the light-absorbing chromophore, followed by slower and more global structural transitions and/or chemical change that ‘encode’ the output response. Natural light-activated systems are ideal for TR-SFX experiments [6] and have many benefits over thermally activated proteins that require technically demanding and constrained methods for reaction initiation, such as the mix-and-inject approach [49,143–145]. A key to success is the combination of time-resolved SFX, solution scattering studies, spectroscopy and computational simulations to access chemistry and dynamics across multiple timescales (fs–s). This is an exciting opportunity and one that will no doubt lead to new and important insights into fundamental aspects of biological catalysis, signal transduction and protein motion.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

All the authors contributed to writing this review.

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