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Protein motions visualized by femtosecond time-resolved crystallography: The case of photosensory vs photosynthetic proteins

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Abstract

Proteins are dynamic objects and undergo conformational changes when functioning. These changes range from interconversion between states in equilibrium to ultrafast and coherent structural motions within one perturbed state. Time-resolved serial femtosecond crystallography at free-electron X-ray lasers can unravel structural changes with atomic resolution and down to femtosecond time scales. In this review, we summarize recent advances on detecting structural changes for phytochrome photosensor proteins and a bacterial photosynthetic reaction center. In the phytochrome structural changes are extensive and involve major rearrangements of many amino acids and water molecules, accompanying the regulation of its biochemical activity, whereas in the photosynthetic reaction center protein the structural changes are smaller, more localized, and are optimized to facilitate electron transfer along the chromophores. The detected structural motions underpin the proteins' function, providing a showcase for the importance of detecting ultrafast protein structural dynamics.

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Most photoactive proteins can be classed into two categories: photosynthetic proteins, which convert solar energy into chemical energy, and photosensory proteins, which sense light and help organisms to adapt and to react to environmental light conditions. Photosensory proteins may guide movement, perception, growth, reproduction, or other functions to enable the survival and the development of the host organism. Examples are rhodopsins [1,2], phytochromes [3,4], or phototropins [5]. Proteins from both classes can work together, for example when photosensory proteins optimize photosynthetic activity [6]. Some protein families, for instance rhodopsins and photolyases/cryptochromes, contain members from both categories. Photosensory proteins are highly interesting templates for protein engineering and have been exploited in microscopy as fluorescent variants [7] and are also used to control cellular responses through optogenetics [*8,9].

Proteins are dynamic objects [10]. The relevant timescales to be studied range from ultrashort femtoseconds to days or even longer. A textbook example of an ultrafast event is the isomerization of retinals in vision [11]. Nano-to millisecond time scales are important for the proton translocation by rhodopsins across the membrane [12], while the kinetics of minutes, hours, or days are relevant for diverse biological actions, such as for photoswitching of sensor protein [13], protection of photosynthetic apparatus from excess light [14], or the assembly and disassembly of intracellular scaffolding proteins [15]. Protein structural changes accompany the function of enzymes on all of these time scales.

Proteins motions on ultrafast femtosecond timescales have been the subject of fascination for a long time. One highly visible, but speculative proposal was that ultrafast protein motions "wire together" different chromophores in antenna proteins, leading to "quantum electronic coherence" in solar energy harvesting [16]. As intriguing as this idea may be, the proposal has been challenged and more reliable interpretations of the complex spectroscopic signals have been put forward [17]. Up to very recently, it has not been possible to directly visualize protein motions on femtosecond time scales. This situation has recently changed when the time-resolution of time-resolved crystallography [18,19] was pushed into the femtosecond regime using serial femtosecond

crystallography (SFX) [20,21]. Now, it can be investigated how ultrafast structural dynamics control chemical reactions of active sites, for example around a chromophore; how structural changes may stabilize transient charges in proteins; and what the protein structure of intermediate and transient states is. The ability to observe these changes directly puts the understanding of protein dynamics on a solid experimental basis.

Before we summarize recent progresses towards visualizing structural changes in two photoactive proteins, we clarify the terms "*kinetics*" and "*dynamics*", which now find entrance to papers in structural biology. Chemical *kinetics* is when rates of chemical reactions are determined. Studying reaction kinetics, for example as a function of environmental changes such as temperature, pH, and substrate concentrations, leads to a deeper and more practical understanding of chemical reactions. It can also lead to the identification of reaction mechanism. With time-resolved crystallography it is possible not only to study the rates between intermediates of the catalytic cycle (Figure 1a), but also to determine the structures of associated transient states from the same set of X-ray data [22–24]. Chemical *dynamics* are concerned with the question of how atoms move within molecules, and how this leads to chemical reactions. Fluctuations of protein- and chemical structures occur all the time and drive chemical changes, but direct observation of these movements is only possible when they are observed in single molecules – which is not feasible at present – or when the movements are synchronized in time over an ensemble of molecules. This occurs at a short time (femtoseconds) after photoexcitation of a photoactive protein (Figure 1d). An example of such coherent dynamics is the direct observation of retinal oscillation in bacteriorhodospin, which finally results in isomerization (Figure 1c) [**25].

In order to measure fundamental events of structural dynamics in proteins, such as the breaking of a bond or the isomerization of a central chromophore, a time-resolution of tens of femtoseconds and a spatial resolution of 3 Å or better is required. The time-resolution is achieved by using optical femtosecond laser pulses in combination with pump-probe measurements, where a short "pump" pulse triggers the reaction and another "probe" pulse arrives at a known time delay to probe the effect of the pump pulse on the sample. The probe can be any electromagnetic pulse, and to achieve the required spatial resolution, the diffraction from ultrashort pulses of X-rays or electrons can be used. Ultrashort X-ray pulses are now available at free-electron X-ray laser facilities. In time-resolved SFX a stream of micrometer-sized crystals is supplied to the interaction point of the X-ray and the optical beams (Figure 1b) [26]. This can be done in various viscous media [27], or in water liquid phase, and also different jet systems have been developed (reviewed in Ref. [28]). The reflection of thousands of crystals is acquired at known delay times after the optical pulse has arrived. The patterns are indexed, scaled, and merged into a structure factor. Difference structure factors are computed by subtracting structure factors from a reference state and transformed into real space for visualization. The observable is the so-called difference electron density map.

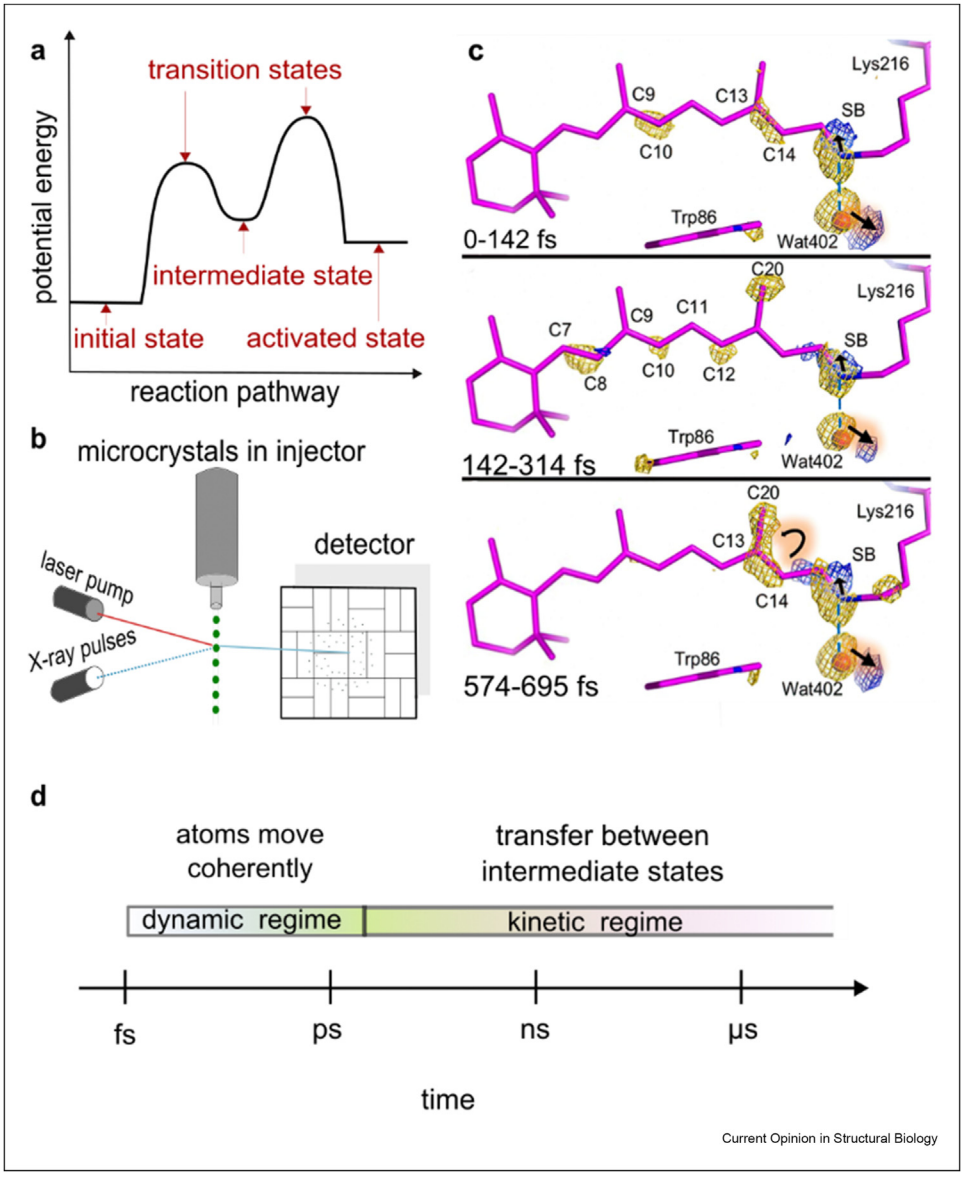
Time-resolved SFX comes with a number of limitations and challenges. When optical laser pulses are used to trigger the reaction, only light active proteins can be investigated. Moreover, it seems that high excitation densities are necessary to obtain good difference signals. The issue has been reviewed recently [29]. High excitation densities can in principle cause multi-photon absorption, which can lead to side reactions and structural changes which are not biologically relevant. To identify potential two-photon absorption the difference diffraction signals should be recorded as a function of excitation photon energy [**30,31], which is however problematic due to the limited beamtime available. Another limitation is that the crystal packing may restrict conformational changes in proteins, especially when large structural changes are probed that change the shape of the protein and which involved residues that form contacts with neighboring proteins in the crystal. Such changes typically occur on nanosecond time scales and later. This limitation is less severe when the changes are confined to the inner part of the protein and when the structural changes to be determined are small in comparison to the size of the unit cell. This is typically the case on ultrafast time scales. It is difficult to know whether a particular structural change is affected by crystal packing, but an assessment along the outlined criteria is helpful. In time-resolved crystallography experiments, the reflection intensities must be measured with high accuracy, placing high demands on the stability of X-ray sources and X-ray detectors. Finally, very little beamtime has been available to users. This challenge has recently been alleviated with the availability of a larger number of XFEL sources.

As a first example, we discuss phytochromes, which are photosensory proteins found in plants, bacterial, and fungi [3,32]. Across the entire superfamily, they consist of a photosensory core module (usually consisting of PAS, GAF, and PHY domains), which host the light-active bilin chromophore, and an additional number of different C-terminal output domains. Phytochromes photo-switch between a red-light absorbing (Pr) state and a far-red light absorbing (Pfr) state, traversing through a number of intermediate states in the photoreaction. The structure of the photosensory core domain of bacterial phytochromes has been known for some time now [33–35], and structures of full-length proteins are starting to be solved [36–38]. A highly curious structural feature of phytochromes is that a conserved part of the PHY-domain (the so-called tongue region)

refolds from a beta sheet in Pr to an alpha helix in Pfr [39]. It is also clear that the isomerization of the D-ring in biliverdin drives the photoconversion, but it is not understood how this change is transduced into the protein environment.

Using time-resolved SFX, we have solved the structure of the photosensory core module (PCM) of a bacteriophytochrome from *Stigmatella aurantiaca* (*Sa*BphP) at 5 ns and 33 ms after photoexcitation (Figure 2a and Figure 2b) [**40]. At the probed time points the protein is in its first ground state intermediate, called Lumi-R. The difference electron density revealed extensive structural changes in almost all parts of the entire phytochrome. This observation may indicate a shift in the dimer arrangement of the

Figure 1



Kinetics vs dynamics and time-resolved serial femtosecond crystallography. (a) The energy diagram of different states during a chemical reaction is shown. Photoactive proteins commonly traverse through a few intermediate states before reaching the activated state. (b) A schematic view of time-resolved SFX. The stream of micrometer-sized crystals is supplied to the interaction point where short pulses of X-ray and the optical laser coincide. Diffraction patterns are collected on a 2D X-ray detector as a function of time-delay between X-ray and optical pulses. (c) Coherent nuclear motions in bacteriorhodopsin as resolved by time-resolved SFX. Trans-retinal isomerizes to cis-retinal (third panel) after coherent oscillatory movement of the retinal photoactivation (panels 1 and 2). Gold and blue changes mark negative and positive differences in electron densities, respectively. The panel was reproduced with permission from Ref. [**25]. (d) Schematic drawing depicting the time scale differences of chemical dynamics and kinetics. The transition time from the dynamic to the kinetic regime is determined by the decoherence of the structural motions in the dynamic regime, which is typically about 1 ps after photoexcitation.

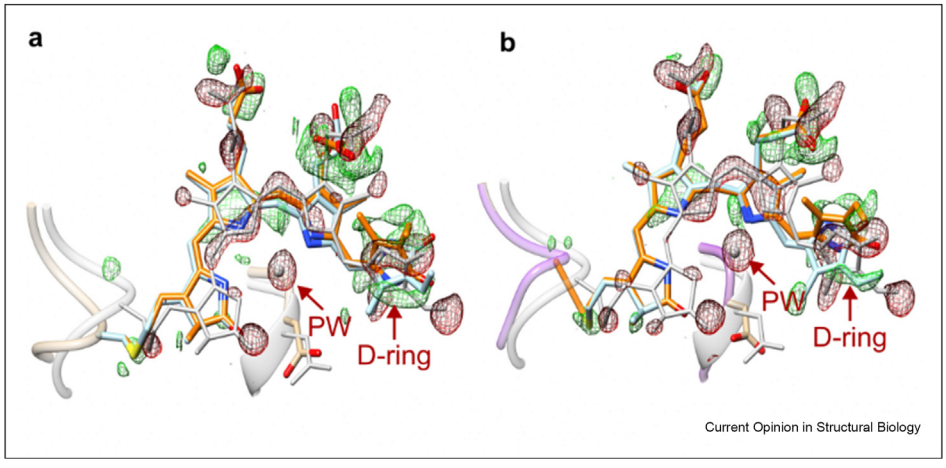
phytochrome. The structure shows that the D-ring in biliverdin is isomerized from 15Z-to 15E configuration at 5 ns and that extensive arrangements have occurred in the biliverdin binding pocket. Changes in the tongue region were not observed, which could be caused by the crystal packing of the region, or because the refolding occurs in a later intermediate state. Overall, the degree of structural changes that was observed is large, indicating that the active state of the protein may have a significantly altered structure compared to the resting Pr state. In *S. aurantiaca* the aforementioned *Sa*BphP regulates the growth of fruiting bodies, which is interestingly the multicellular state of this prokaryotic organism [41,42].

Elucidating how the Lumi-R state is formed from the Pr* state, a structural snapshot of the PAS-GAF fragment of a *Deinococcus radiodurans* phytochrome (*Dr*BphP) was solved at 1 ps after photoexcitation [**30]. The snapshot confirmed directly that rotation of the D-ring is involved in the primary reaction coordinate of the chromophore (Figure 3b). This had been proposed for decades, and is now confirmed by direct structural observation. Further, the structure showed extensive rearrangement of the chromophore ring A, and C, and even of parts of the bilin which are not within the π-conjugated system (Figure 3c). The structure of the entire chromophore binding pocket was rearranged, consistent with that it has to loosen up for the biliverdin to be able to isomerize (Figure 3c). The most surprising change was the hydrolysis of the so-called pyrrole water, which is found in all crystal structures in the center of the chromophore (Figure 2d) [33–35,39,40,42]. The chemical implications of these changes are not yet fully explored, and motivate further investigations with time-resolved structural techniques. In *D. radiodurans*, the aforementioned *Dr*BphP is a light-gated phosphatase [*43].

Compared to other light-sensitive proteins [20,44–48], phytochromes show a very high number of structural changes already at a ps after photoexcitation. Changes can be observed not only on the level of amino acids that accompany the conformational changes of the biliverdin, but also on all the water molecules that surround the chromophore. The importance of water molecules found with time-resolved SFX is consistent with a recent femtosecond infrared spectroscopy study on a bathy phytochrome, where transient deprotonation of the biliverdin into a nearby water cluster was found upon photoactivation of the Pfr state [*49]. Active waters are not unique to phytochromes and a water near the Schiff base in bacteriorhodopsin is also active on the femtosecond time scales (Figure 1c) (Nogly et al., 2018). Water is important for the structure and dynamics of proteins [50], and now this can be observed directly on femtosecond time scales. The broad and delocalized response of the binding pocket in phytochromes is consistent with that many amino acids can be altered by mutation to modify, but not completely block, the proteins' photoactivity [51]. The chromophore binding pocket of phytochromes offers a lot of flexibility for fine-tuning the signaling capacity of the protein in different organisms.

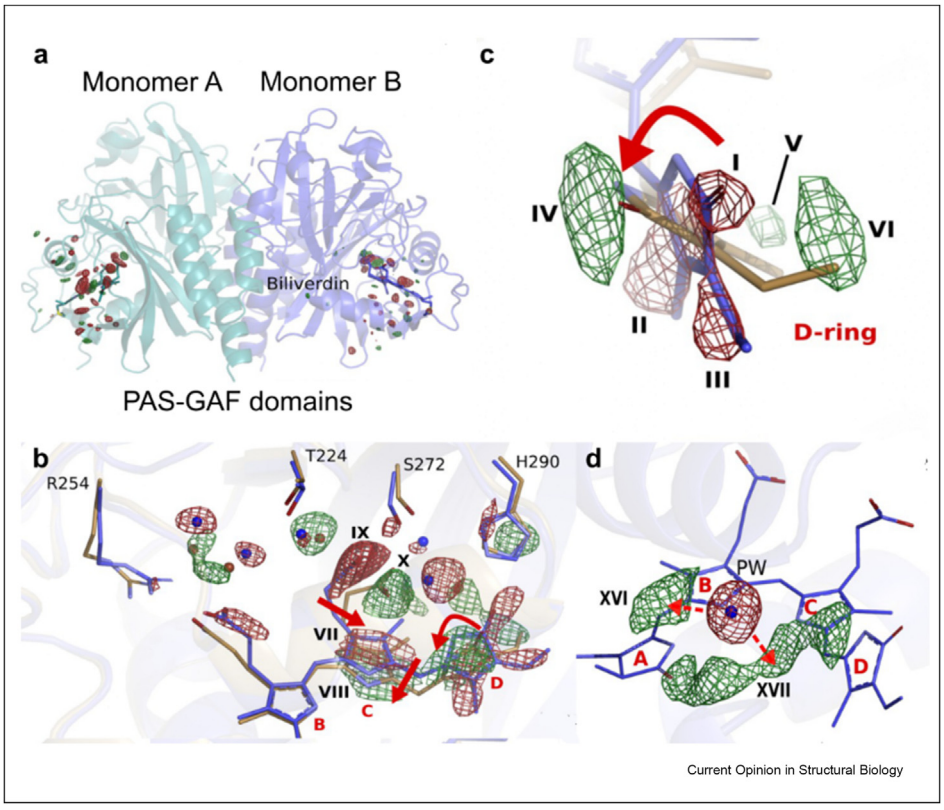
Having discussed structural changes in *photosensory* phytochromes, we will now review a study on ultrafast structural changes in the *photosynthetic* reaction center protein of *Blastochloris viridis* [**52]. The reaction center is a dimeric membrane protein, which holds a number of bacteriochlorophyll cofactors. These accept light energy by direct absorption and from the light harvesting

Figure 2



Structural changes of phytochrome intermediates. (a) and (b) show the difference electron density map around the chromophore region of the phytochrome from *S. aurantiaca* (PAS, GAF, PHY domains), at 5ns and 33 ms after photoexcitation, respectively. Green and red densities mark positive and negative electron densities in all panels and the maps are contoured at 2.7 Sigma. The gray structure is for the dark/reference state and the orange structure is for the fully isomerized chromophore; the blue structure is for a partially isomerized intermediate [**40]. The domain names are cGMP phosphodiesterase-adenylate cyclase FhlA (GAF), Per-ARNT-Sim (PAS), and phytochrome-specific (PHY).

Figure 3



Ultrafast structural motions in phytochromes. (a) The difference electron density map contoured at 4.5 sigma on the PAS-GAF domains of the phytochrome from *D. radiodurans* is shown overlayed over the *Dr*BphP PAS-GAF dimer (shown in light blue and blue). (b) The observed difference electron density contoured at 3.3 Sigma with refined *Dr*BphP$_{dark}$ (blue) and *Dr*BphP$_{1ps}$ (beige) structures is shown for the surrounding of the B-, C-, and D-rings. (c) The same maps and structures are shown around the D-ring of biliverdin chromophore. (d) The same difference electron density is shown at the site of photodissociation of the pyrrole water, contoured at 3.3 sigma. Green and red surfaces mark positive and negative difference electron densities, resepctively, in all panels. The panels were reproduced with permission of Ref. [**30].

complexes. The energy is then transferred to two bacteriochlorophylls, which are called the "special pair", and where charges are generated within a few picoseconds. The charges are then transported to the opposite site of the membrane. The study is the first to probe the ultrafast structural responses of a plant or a bacterial photosynthetic reaction center. Structural snapshots at 1 ps, 5 ps, 20 ps, 300 ps, and 8 µs indicated a series of difference electron density peaks (Figure 4), which were localized on the active chromophores and a few neighboring amino acids (Figure 4f). The center of attention is the two bacteriochlorophylls of the special pair, where charge separation occurs. A consistent and strong positive peak indicates that the pair moves closer together after charge generation (Figure 4a–e). This feature is strongest at <20 ps, smaller at 300 ps, and has decayed at 8 µs. Associated changes are observed around two histidines in the L-subunit and a histidine and tyrosine in the M-subunit (Figure 4e). The same study also reported low-amplitude motions of several transmembrane helices in the reaction center protein, which are currently unaccounted for in theory.

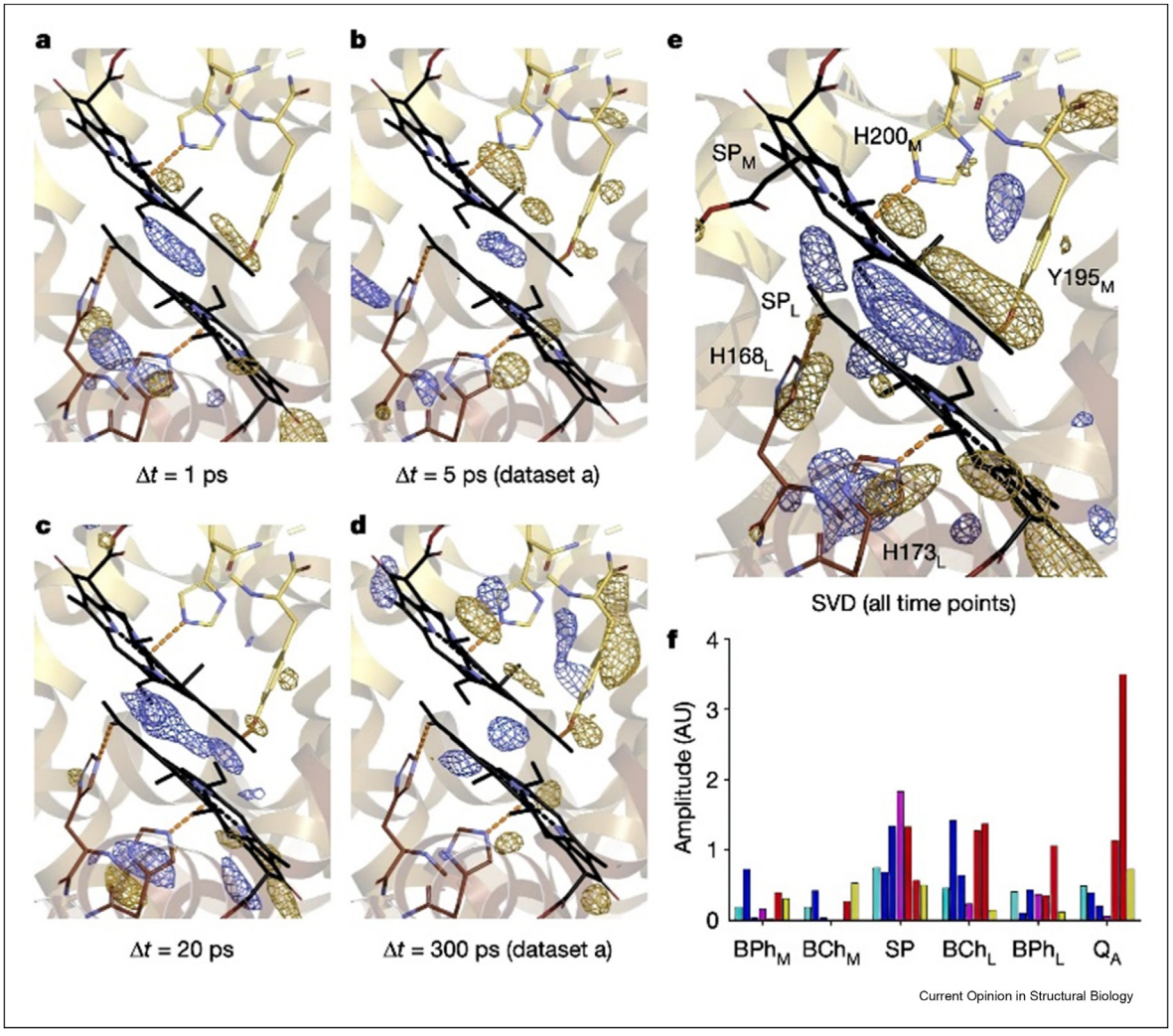
The time-resolved SFX data strongly suggest that the reaction center protein primarily acts like a rigid scaffold to hold the chromophores in place during charge separation, but that it also undergoes subtle rearrangements to stabilize the charges while they traverse through the protein. Amino acids close to the charged chromophores adjust to stabilize them, but only to a degree that is suitable for charge transfer to the next chromophore. Overall, the structural changes are much smaller than in phytochromes, but similar in magnitude to structural changes observed in plant photosystems [46,47].

Here, we have reviewed the structural changes of two proteins belonging to the categories of photosynthetic and photosensory proteins. We propose that the degree of ultrafast and transient structural changes is optimized by evolution as to support the specific function of the enzymes: In the photosynthetic reaction center, the protein motions are optimized for maximally efficient charge generation and separation across the membrane. For swift and unidirectional electron transfer the protein provides a stable scaffold, which primarily holds the chromophores

in place. Further, it allows for structural changes of the chromophores, for some dynamic rearrangements of neighboring amino acid side chains, and for some small helices movements. In the photosynthetic proton pump bacteriorhodopsin, the ultrafast structural changes are larger, owing to the fast isomerization reaction of the retinal cofactor [**25,31]. In phytochromes, changes across the binding pocket and the entire protein are extensive, underpinning the plasticity and flexibility of the photoreaction. Here, the maximal efficiency of the photoreaction is not crucial. Rather, the broad response is a sign that the protein has evolved so that the photoreactions of the phytochrome can be tailored to the sensing needs of the host organism. Thus, from a functional point, it is highly reasonable that the binding pocket is a very active player in the photoreaction, so that many residues can be changed by evolution to fine-tune the isomerization reaction. In all cases, the extent of ultrafast structural changes underpin the function of the proteins.

Discovery of ultrafast proteins motions has just begun, and more snapshots of activated proteins are expected to be published in the coming months and years. It will be important to address the current challenges of time-resolved SFX, in particular the high excitation densities that are required to obtain difference electron density signals [31] and the inaccuracies in amplitude determination inherent to the highly partial type of reflection intensities collected with each diffraction pattern. This will require technical advances, such as stable and

Figure 4



Light-induced electron density changes in a bacterial reaction center at the site of photo-oxidation. (a)–(d) The time points for the maps are indicated in the figure. (e) The principal component from SVD analysis of all the experimental difference Fourier electron density maps. All maps are contoured at ±3.2 sigma. Blue and gold represent positive and negative difference electron density, respectively. f) Relative amplitudes of difference electron density features integrated within a 4.5 Å sphere centered on the cofactors of the reaction center. The color bars represent (from left to right): cyan, Δt = 1 ps; blue, Δt = 5 ps, datasets a and b (in that order); purple, Δt = 20 ps; red, Δt = 300 ps, datasets b and a (in that order); mustard, Δt = 8 μs. The figure and parts of the legend were reproduced with permission from Ref. [**52].

accurate detectors, higher repetition rates of the X-ray lasers, new ways of supplying the microcrystals to the x-rays [53], more streamlined data analysis schemes [54], and advanced approaches to filter relevant information out of the data [55]. Within the next decade, a lot of new insight into ultrafast motions in proteins will arise, boosting our understanding of biochemical reactivity.

Conflict of interest statement

Nothing declared.

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

* of special interest
** of outstanding interest

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