BIOLOGICAL, MACROMOLECULAR SWITCHES AND CYCLERS

ANDREW T. ULIJASZ1, NAMRTA PURWAR2, VUKICA SRAJER3, MARIUS SCHMIDT2*

1University of Wisconsin-Milwaukee, Department of Biological Sciences, 3209 N. Maryland Ave. Milwaukee, WI, 53211, USA
2University of Wisconsin-Milwaukee, Department of Physics, 1900 E. Kenwood Blvd. Milwaukee, WI, 53211, USA
3University of Chicago, Consortium for Advanced Radiation Sources, 5640 S. Ellis Avenue, Chicago, IL 60637, USA

(Received July 21, 2010)

Here we present preliminary results on the time-dependent absorption responses of two macromolecular photoswitches towards light. Unlike photocyclers such as the photoactive yellow protein, photoswitches form distinct, thermally stable species that differ in their absorption spectra. They can be shifted almost indefinitely back and forth between these two stable states. Investigations on a cyanobacterial phytochrome as well as on a phycobiliprotein are shown. These initial results demonstrate that we may better understand the switching reaction that occurs in such chromoproteins through highly advanced data collection methods.

Key words: time-resolved spectroscopy, protein crystallography, transient state kinetics, chemical kinetics.

INTRODUCTION

Chemical reactions catalyzed by proteins lie at the base of life. Enzyme kinetics as taught in textbooks is essentially chemical kinetics (1). Catalysis is a multi-step process. In a more complex catalyst such as an enzyme, these steps require substantial reorganization of the structure of the catalyst on the atomic level (2, 3). Each step may be accompanied by a multitude of different configurations and conformations. Steady state kinetics (4) parameterizes the complexity of the enzyme’s conformational space by a single value, the Michaelis coefficient. In order to investigate the catalytic process in all complexity, transient state kinetics must be employed. Typically, methods are of the pump probe type or consist of rapid, stroboscopic measurements. They all aim at determining the rate coefficients that make up the mechanism, plus the physical properties of the intermediate states.

*Corresponding author (Email: m-schmidt@uwm.edu; Phone: +1-414-229-4338)

ROM. J. BIOCHEM., 47, 2, 155–164 (2010)
The former determine the kinetics and the latter give information on, in the widest sense, the structure of the protein that catalyses the reaction. Time-resolved crystallography (2, 5, 6) tackles this problem in the boldest fashion, since it unifies kinetics with structure determination on the atomic length scale (~0.2 nm). X-ray crystallographic data can be collected at a synchrotron as rapid as 100 ps using the Laue technique (Fig. 1). The chemical reaction in the protein crystals can then be followed from the beginning to the very end of the photocycle, with a succession of electron density maps sequentially arranged similar to a movie format.

Fig. 1. – Laue image of a protein crystal. The crystal is held still during the exposure with X-ray pulses of 100 ps duration.

From this movie of electron density values the structures of the intermediates and information about the kinetics can be extracted (7–16). Of particular importance is the singular value decomposition of the time-resolved X-ray data (9, 10, 14, 15, 17), as it separates time from space variables and ultimately allows for the simultaneous determination of the intermediate structures and kinetics.

It has been shown (10, 17) that one time-series of time-resolved X-ray data is not usually sufficient to determine a mechanism comprehensively with all of its rate coefficients. This is because the number of rate coefficients by far exceeds the number of observable relaxation times. In crystallography, concentration is equivalent to electron density. There is no linear factor in between as is the case, for example, in absorption spectroscopy where the absorption coefficient is usually
an unknown variable. Crystallography disposes additional observables to determine the kinetics (9), but even then, a single time-series is not sufficient (17). An additional parameter must be varied, the most convenient of which is the temperature. Thus, in such a scenario crystallography would become five-dimensional, by simultaneously collecting time, space and temperature data. Ultimately with this method, a comprehensive mechanism can be determined, including enthalpy, entropy and pre-factors of all rate coefficients and, most importantly, a set of largely refined atomic structures of short-lived intermediates.

There are a number of interesting biological systems that are potentially suited to be subject to 5-dimensional crystallography. The most suitable employ a chemical reaction that can be triggered repeatedly by light. Hence they employ either photocycles or resemble photoswitches, a process often referred to as photoconversion.

PHOTOCYCLERS AND PHOTOSWITCHES

Typical photocycles are exemplified by both bacteriorhodopsin (18) and photoactive yellow protein (PYP) (19). PYP (Fig. 2) is ideal for time-resolved crystallographic applications, as it forms strongly diffracting, bright yellow crystals that are particularly insensitive to radiation damage. The PYP photocycle can be easily triggered by pulses of blue light around $\lambda=480$ nm. The central chromophore, 4-hydroxy-cinnamic acid (see also Fig. 2), rapidly isomerizes from trans to cis, driving the photocycle which consists of at least 6 intermediates (14). These intermediates form and decay on time-scales ranging from pico-seconds to seconds. For more information, see Rajagopal et al. (15) and Ihee et al. (14).

Fig. 2. – Crystal structure of PYP. The central chromophore 4-hydroxycinnamic acid (HC4) is shown as well as Arg52 which swings open upon isomerisation of HC4.
Photooptical switches are in stark contrast to photocyclers in that they do not only inadvertently revert back to their dark-adapted ground state after switching to the light-driven excited state, but instead adopt distinct and stable configurations that differ in their absorption spectra. The most prominent members of the photo-switch class are the Phytochromes (Phys) (20), bilin-containing red far-red reversible photoreceptors discovered in plants over 60 years ago for their critical roles in plant behaviour, development and general agriculture importance. Specifically, the Phy chromophore, phytochromobilin, can be photoverted by red light from a species that absorbs in the red, or Pr, to one that absorbs in the far-red, or Pfr. For plant Phys, the absorption spectrum typically shifts from 680 nm to 720 nm, respectively. Like PYP and other light receptors, Phys undergo several intermediate states during photoconversion, ranging from picosecond to millisecond timescales (Fig. 3) (21). In the dark, Phys in the excited state (Pfr) will slowly thermally decay back to the Pr dark-adapted state, a presently ill-defined process known as “dark reversion” (Fig. 3).

More recently, Phys have been found in a plethora of microbes (22–24) and due to their stability have been more amenable to crystallization than plant versions. Consequently, their structures can be determined. In contrast to plant Phys, the bacteriophytochromes found in eubacteria and cyanobacterial phytochromes (Cphs) found in cyanobacteria contain alternative chromophores. For their photoactive pigments bacteriophytochromes use biliverdin (BV), the immediate heme breakdown product catalyzed by heme oxygenase, whereas cyanobacterial versions use phycocyanobilin (PCB), catalyzed by an additional enzyme that
reduces BV. Recent data suggest that Phys isomerisation and general mechanisms might differ greatly between phylogenetic clades (3, 24). In some Phys ring D of the chromophore is thought to isomerize about the double bond (25–27), in others ring A movement is predicted to initiate subsequent events (Fig. 4) (3). The canonical isomerisation reactions that occur in these bilins are called \( Z \) to \( E \)-isomerizations (standing for the German words zusammen and entfernt, respectively). In the case of Phys, the isomerization and/or chromophore displacement then initiates movements of the light-sensing domain of the protein that eventually signal through an “output domain” to downstream effectors, culminating in a behavioural alteration of the organism (3).

There are other biliproteins that also display \( Z \) to \( E \)-isomerizations that are not within the Phy superfamily. The \( \alpha \)-phycoerythrocyanin (PEC) found in the phycobilisomes of some photosynthetically active cyanobacteria is a prime example. PEC was the first biliprotein whose structure was determined with the chromophore in the \( E \)-configuration (28). Together with the previously determined structure of the \( Z \)-form (29, 30), a \( Z \) to \( E \)-isomerization could be characterized crystallographically by the static structures of the two flanking states (Fig. 5). However, it is currently unclear exactly how the structures transform during the photoconversion reaction. \( \alpha \)-PEC in its \( E \)-configuration forms relatively stable crystals (Fig. 6) that need to be kept under protecting light (630 nm) or in the dark and may be easily photoconverted within the crystals from the \( E \)- to \( Z \)-configuration. Hence, these crystals are well suited for time-resolved spectroscopic investigations using a fast time-resolved micro-spectrophotometer (31).
MATERIALS AND METHODS

PEC was isolated from phycobilisomes of *M. laminosus* as reported (32). The \( \alpha \)-subunit was separated by osmotic shock and purified by anion chromatography. Pure protein was obtained by a final size exclusion chromatography step using Sephadex G75. The \( \alpha \)-PEC was concentrated to 10 mg/ml and crystallized with potassium-phosphate and MgSO\(_4\) as reported by Schmidt *et al.* (28). The crystallization trays were irradiated for one hour with yellow light (~580 nm) before crystallization was achieved in the dark.
Recombinant SyB-Cph1 GAF-Phy construct chromoprotein was cloned in expression plasmid pBAD-6H and expressed using the dual plasmid system as described previously (33).

Microspectroscopic investigations on the α-PEC crystals were performed in the Cryolab at the ESRF (31). A crystal was looped under protecting light (\(\lambda=630\) nm) and quickly measured at 0°C using the white light of a Xenon light source as measuring light.

Nanoliter sized droplets of the phytochromes were measured in capillaries at the microspectrophotometer facility at BioCARS, Advanced Photon Source, Argonne Nat’l lab. The temperature was set to 55°C by a Cryostream (Oxford cryosystems). To shift the phytochrome between its respective Pr and Pfr forms, filtered light of either 630 nm or 690 nm were respectively applied. The spectra were quickly measured using a microspectrophotometer (4DX) equipped with a Xenon light source and a fibre spectrometer (both Ocean optics). The Xenon light source intensity was substantially dimmed to prevent the photoreaction from occurring.

RESULTS

Fig. 6 shows the spectra obtained from crystals of the α-PEC crystallized in the E-form. The white measuring light drives the transition from E- to Z in the crystals, so that after 6 minutes (250 s) the spectrum of the Z-configuration is reached (compare insert: solution spectra).

![Fig. 7. – Absorption and difference absorption spectra for the phytochrome. Cph1 Pr: SyB-Cph1(GAF-Phy) Pr spectrum, Cph1 Pfr: SyB-Cph1(GAF-Phy) Pfr spectrum. Spectra marked 1s to 200s: difference Pfr-Pr spectra from 1s to 200s (arrow) after the 630 nm light is shut off. Insert: time-dependence of the Pfr-Pr difference spectra. Bullets: summed absolute differences between difference spectra at time t and the one at time 1s. Solid black line: fit by exponential function.](image-url)
The SyB-Cph1 photochrome can be shifted from its red-absorbing Pr form (marked Cph1 Pr in Fig. 7) to its far-red-absorbing Pfr form (marked Cph1 Pfr in Fig. 7) by illuminating the solution for a few minutes at 630 nm. Vice versa, Pfr can be shifted back to Pr by illuminating with 690 nm light. This process can be repeated almost indefinitely. In Fig. 7 the difference spectra Pfr minus Pr are also shown. Directly after the 630 nm light was removed, difference spectra were collected at a rate of approximately one per second. In the first few seconds the difference spectra have clearly changed (indicated by the black arrow) only to remain constant for the rest of our measurement. The difference between the spectra is plotted as a function of time in the insert. The black curve is an exponential function fitted to the data with a relaxation time $\tau$ of 0.6 s.

DISCUSSION

Our results described here demonstrate that both $\alpha$-PEC and the SyB-Cph1 Phy show clear responses to illumination with different wavelengths of light. For the SyB-Cph1 phytochrome, we observed promising preliminary results with time-resolved absorption spectroscopy, showing a possible intermediate forming in the second timescale. The kinetics of these responses now needs to be more rigorously investigated (e.g., in nano-millisecond timescales) to enhance our understanding of how these molecular switches function in detail. Now that both ground and excited state structures of a representative family member from the phycobilisomes ($\alpha$-PEC) and phytochromes (SyB-Cph1) are available, it is possible to more accurately tease apart the timing of the chromoprotein movements between these two end states using several physico-chemical techniques. Specifically, methods to investigate transient states such as time-resolved absorption spectroscopy (used in this study) or, ultimately, time-resolved X-ray crystallography are ideally suited to answer questions regarding the kinetics of the switching reaction and the number of potential intermediate states involved. Moreover, information about the atomic structures of the intermediates can be obtained. These investigations will lead to a more detailed description of the mechanism of light-driven molecular switching in general. Potential switching motifs are likely to be detected and may be engineered into other coloured proteins to transform them into optical switches for numerous applications in molecular and cellular biophysics.

Acknowledgements. The use of the BioCARS Sector 14 was supported by the National Institutes of Health, National Center for Research Resources, under grant number RR007707. M.S. is supported by the National Science Foundation, grant number MCB-0952643 (CAREER). A.U. and M.S. are supported by the college of L&S, UW-Milwaukee.
REFERENCES