The Speed of Allosteric Signaling Within a Single-Domain Protein

Olga Bozovic, Jeannette Ruf, Claudio Zanobini, Brankica Jankovic, David Buhrke, Philip J. M. Johnson, Peter Hamm*  
Department of Chemistry, University of Zurich, Zurich, Switzerland  
*peter.hamm@chem.uzh.ch

While much is known about different allosteric regulation mechanisms, the nature of the “allosteric signal”, and the timescale on which it propagates, remains elusive. The PDZ3 domain from postsynaptic density-95 protein is a small protein domain with a terminal third alpha helix - the $\alpha_3$-helix, which is known to be allosterically active. By cross-linking the allosteric helix with an azobenzene moiety, we obtained a photocontrollable PDZ3 variant. Photoswitching triggers its allosteric transition, resulting in a change in binding affinity of a peptide to the remote binding pocket. Using time-resolved infrared and UV/Vis spectroscopy, we follow the allosteric signal transduction and reconstruct the timeline in which the allosteric signal propagates through the protein within 200 ns.

I. INTRODUCTION

To adapt is to survive, and all living cells need to constantly alter their activity according to the evolving conditions of their surroundings in order to preserve homeostasis. Allosteric regulation is one of the main tools utilized by Nature to alter a target molecules’ activity by adjusting its affinity to certain substrates as a response to an incoming signal. It represents the communication between two non-overlapping sites of a protein and is used as a mechanism for regulating activity. However, the very nature of the allosteric signal is not clear. Within the framework of traditional structural biology, transduction of the allosteric signal has long been considered to be the propagation of structural change throughout oligomeric proteins. The concept of dynamic allostery has been introduced as well, where allostery is transduced by changing the flexibility of certain parts of a protein, and thereby its entropy, without major structural rearrangements. Very recently, long-ranged charge re-organisations have been proposed as yet another mechanism of allostery.

Allostery is often related to the communication between the domains of larger, multi-domain proteins. With the discovery that isolated PDZ domains have allosteric properties as well, they became attractive systems to study alloster within single, small domain proteins. PDZ domains are a prime example, in which allostery is thought to be driven by changes in the intrinsic protein dynamics. The PDZ3 domain from PSD-95 protein (Post Synaptic Density-95) differs from the rest of the PDZ family by an auxiliary helix at its C-terminus, and is particularly interesting in this regard. Petit et al. showed that even though this $\alpha_3$-helix does not form any direct contact with the peptide ligand, its deletion dramatically decreases the binding affinity of the ligand to the protein by 21-fold. In our previous work, we designed a photocontrollable variant of the PDZ3 domain, showing that allostery could be controlled by light by photoswitching the allosteric element - the $\alpha_3$-helix. To that end, an azobenzene moiety has been covalently linked to the $\alpha_3$-helix in a way that photo-isomerisation of the former stabilizes/destablizes the helicity of the latter, as an analogue of a process observed in vivo, where the phosphorylation of residue tyr397 has the same effect. The construct (see Fig. 1) is arguably the smallest truly allosteric protein with clearly identifiable allosteric and active sites. We were able to allosterically perturb the binding affinity for the peptide ligand by photoswitching the distal helix with astonishing changes in binding affinities, up to 120 fold depending on temperature. Conversely, the rate of the photoswitch’s thermal cis-to-trans isomerisation is influenced by the presence or absence of the peptide ligand. By measuring binding affinities at different temperatures, we were able to construct a thermodynamic cycle of allostery and quantify the allosteric force transmitted from the peptide ligand through the PDZ3 protein to the photoswitch.

Here, we exploit the structural sensitivity of time-resolved infrared spectroscopy combined with time-resolved UV/Vis spectroscopy, to follow the allosteric sig-
nal in the photoswitchable PDZ3 domain from the point of its origin – the α3-helix - through the protein until it reaches the binding groove and finally transduces to the peptide. We reveal the progression of the signal that follows the allosteric event and show that there is a well-defined temporal sequence of events, despite the fact that the protein responds on many timescales.

II. RESULTS AND DISCUSSION

For a spectroscopic signature specific to the source of the allosteric signal, we start with transient UV/Vis spectroscopy of the π-π*-transition of the photoswitch molecule, located on the α3-helix. To that end, the sample was first prepared in the cis-state by constant illumination with a 370 nm cw laser, which promotes trans-to-cis isomerisation of the photoswitch. The purity of the cis-sample obtained in this way is estimated to be above 85%.

Using a short pump pulse at 420 nm, we then induce the ultrafast cis-to-trans isomerisation of the photoswitch, which destabilises the helical structure of the α3-helix. By following the time-resolved response of the π-π*-transition at 370 nm, we can retrieve information on the timescales of photoswitching as well as the subsequent α3-helix unfolding. Thus, we utilize the photoswitch molecule not only as a trigger to perturb the helical structure of the α3-helix, and thereby initiating the allosteric response, but also as a local reporter of photoswitch conformation and α3-helix structure.

The corresponding kinetic traces are shown in Figs. 2a,b for the isolated protein and the protein-peptide complex, respectively. The timescales contained in these data are determined by fitting them to a multiexponential function:

\[ S(t) = a_0 - \sum_k a(\tau_k) e^{-t/\tau_k}, \]  

where a maximum entropy method has been applied for regularisation. In this fit, the timescales \( \tau_k \) were fixed and equally distributed on a logarithmic scale with 10 terms per decade, while the amplitudes \( a(\tau_k) \) were the free fitting parameters. The resulting lifetime spectra \( a(\tau_k) \) are shown in Figs. 2a,b as black lines. In both cases, they reveal two similar kinetic events, one at <100 ps and a second one around 4 ns for the protein-peptide complex and 6 ns for the isolated protein.

The actual isomerisation of the azobenzene moiety around its central N=N bond is a barrier-less picosecond process giving rise to the first peak in the lifetime analysis (the time-resolution of the experiment, 60 ps, does not allow us to time-resolve this process, see Methods). Since the photoswitch is covalently linked to the α3-helix, it will however be in a very constrained environment immediately following isomerisation. It has been shown that the π-π*-band is a sensitive probe of any strained conformation of the photoswitch. We therefore attribute the second kinetic component to the release of that strain, and thus to the partial unfolding of the α3-helix. The time constant, 4 or 6 ns, respectively, is a reasonable value for the forced unfolding of an α-helix. The process speeds up a little bit when a peptide ligand is present in the binding pocket, as a result of the allosteric force transduced towards the photoswitch. Unfolding of the α3-helix represents the starting point of the allosteric signal.

To identify a spectroscopic signature of the addressee of the allosteric signal, the peptide ligand, we compare FTIR difference spectra of the isolated protein (Fig. 3a, purple) with that of the protein-peptide complex (Fig. 3b, green). Each of these signals represent difference spectra upon photoswitching the samples from the cis- to the trans-state after effectively infinite time. There are prominent absorption changes in the amide I region between 1600 and 1700 cm\(^{-1}\), arising mainly from backbone C=O modes of the protein and the peptide, which are known to be strongly structure dependent.

When analyzing the response of the isolated protein, the amide I band represents the conformational rearrangements of the protein upon photoswitching the α3-helix. In contrast, in the protein-peptide complex, this band is the combined response of the protein and the peptide. Assuming that the spectra are additive, one can...
The protein responds to the perturbation induced by the photoswitch with motions over a set of discrete timescales ranging from 1 ns to 10 µs (see Figs. 4a,b), in much the same way as observed for the PDZ2 domain.22 We have attributed this results to a redistribution of population between a set of conformational substates, whose populations shifts upon the allosteric perturbation, in accordance with one of the more recent views of allostery.23 We have attributed this results to a redistribution of population, along the lines of a Markov State Model.22 Such a network picture would not necessarily result in a “sequence of events”. Nevertheless, when isolating the response of the peptide ligand, the dynamical content clears up significantly (compare Fig. 4b vs Fig. 4c), and reveals such a sequence of events. That is, the α3-helix unfolds on a 4 ns timescale (Fig. 4a,b), and it takes 200 ns until that signal arrives at the peptide ligand (Fig. 4c). The more complicated response of the protein tends to mask this process; in fact the 200 ns peak in the dynamical content of the peptide ligand (Fig. 4b) is only a minor contribution to that of the peptide/protein complex (Fig. 4b). The 200 ns timescale establishes the speed of the allosteric signal through the PDZ3 domain.

It is important to stress that the appearance of the allosteric signal at the peptide ligand after 200 ns does not yet result in its unbinding. First, the off-rate, which can be estimated from the binding affinity and typical on-rates for the PDZ3 system, is expected to be by many orders of magnitudes slower, 200 ms. Second, in order to distill out the structural response of the peptide ligand, we have performed these experiments at room temper-
FIG. 4. Dynamical content of (a) the isolated protein, (b) the protein-peptide complex and (c) the peptide ligand, calculated from Eq. 2. The various timescale peaks are labeled according to their major origin.

III. CONCLUSION

Following the dynamics of proteins in real-time requires an instantaneous trigger for the non-equilibrium transition in question, i.e., preferentially a photo-responsive element that enables very high time-resolution. Naturally abundant photoreactive proteins such as photoreceptors and haemoglobin/myoglobin derivatives enable photoinduced perturbations and subsequent time-resolved studies. Unfortunately, the vast majority of proteins are not photo-responsive and need an artificial photoactive element incorporated into their structure. The emerging field of designing artificially photocontrollable systems allows one to introduce the aspect of time in the investigation of fundamental mechanisms of biomolecular recognition.

A sequential propagation of the allosteric signal is somewhat naively thought to be only possible in large multidomain proteins, which undergo a significant conformational rearrangement. As is the example with haemoglobin or ATP synthase, it is natural to picture a large conformational change, which triggers a cascade of events that finally lead to an allosteric effect. However, when discussing allosteric propagation through small protein domains, and especially ones where allostery is predominantly dynamically driven, it becomes unclear what the underlying molecular mechanism is, and what the signal sequence is.

By incorporating the photoswitch moiety to the α3-helix of the PDZ3 domain, we were able to allosterically alter its affinity towards the peptide ligand. Setting proteins in motion by photoswitching, we followed the response of the protein and its ligand in both UV/Vis and infrared spectral regions. Transient measurements exposed that the allosteric signal is transduced in a sequence of events, starting from the point of the perturbation—the α3-helix—through the protein to the binding groove in 200 ns. The 200 ns timescale represents the speed of the allosteric signal within an isolated protein domain.

A time-dependent evaluation of biological processes is necessary in order to reconcile the structure-function relationship of proteins. While a static structural depiction gives us insight into the protein’s function, a broader kinetic picture is needed to comprehend the true dynamical nature of proteins, and capture intermediates that may not be obvious in equilibrium. Revealing the timescales in which signals travel through biological systems will help understanding how changes in intrinsic structure and dynamics ultimately affect and alter their natural function.

IV. METHODS

A. Protein and Peptide Preparation

Photocontrollable PDZ3 domain and KETWV peptide were produced as described previously. The purity of all samples was confirmed with mass spectrometry. The concentration of protein sample was determined by the visible absorption of the photoswitch molecule at 325 nm, assuming an extinction coefficient of $\epsilon = 10,000 \text{ M}^{-1}\text{cm}^{-1}$. The concentration of the peptide was determined by the tryptophan absorption at 280 nm. The concentration of all samples was cross-confirmed with amino acid analysis.
B. Time Resolved Spectroscopy

The time-resolved Vis pump-IR probe and Vis pump-Vis probe experiments were performed using two electronically synchronized Ti:sapphire laser systems with a repetition rate of 2.5 kHz, allowing for delay up to 42 μs [26]. The wavelength of the pump laser was tuned to 840 nm so that second harmonic generation in a BBO crystal produced pump pulses centered at a wavelength of 420 nm to induce cis-to-trans isomerization. To minimize sample degradation, the compressing stage after light amplification was bypassed, resulting in stretched pulses of ca. 60 ps duration FWHM and a power of 4.2 μJ. The beam diameter was ≈ 135 μm FWHM at the sample position. The pump pulses were mechanically chopped at half the repetition rate of the laser setup. The mid-IR probe pulses were obtained in an optical parametric amplifier [27] and centered at 1640 cm⁻¹ with a pulse duration of ≈ 100 fs and a beam diameter of ≈ 115 μm. After the sample, they were passed through a spectrograph and detected in a 2×64 MCT array detector with a spectral resolution of ≈ 2 cm⁻¹/pixel. The water vapor lines of the unpurged setup were used for frequency calibration. The polarization of the pump pulses was set to magic angle (54.7°) relative to the probe pulses and multichannel referencing as described in Ref. [28] was used for noise suppression.

The visible probe experiments were performed as previously described [29]. The probe pulses were obtained by focusing ≈ 1 μJ of the 800 nm probe laser into a CaF₂ window, which was continuously translated.

The samples (≈ 700 μL; 1.1 mM protein and 4.4 mM peptide) were cycled through a closed system with a sample reservoir, a peristaltic pump and a flow-cell, all under inert atmosphere (N₂). The reservoir was illuminated with a continuous wave laser at 370 nm (150 mW, CrystaLaser) to prepare the sample in the cis-state. The sample cell consisted of two CaF₂ windows separated by a 50 μm Teflon spacer. The flow speed of the peristaltic pump was adjusted to ensure sufficient sample exchange before the subsequent laser shot but to minimize flowing out of the sample at the latest time delay (≈ 42 μs). Static reference spectra were acquired in an UV/Vis (Shimadzu UV-2450) and an FTIR (Bruker Tensor 27) spectrometer, respectively, with sample parameters that were comparable to those in the time-resolved experiments.

Acknowledgement: We thank Gerhard Stock and his group for many insightful discussions, the Functional Genomics Center Zurich, especially Serge Chesnov and Birgit Roth, for their work on the mass spectrometry and amino-acid analysis, and Livia Marie Müller for her contribution at an early stage of the project. The work has been supported by the Swiss National Science Foundation (SNF) through the NCCR MUST and Grant 200020B_188694/1.

Data Availability: The data that support the findings of this study are openly available in Zenodo (the link will be provided at the proof stage.)

References:

1. Wodak, S. J. et al. Allostery in its many disguises: From theory to applications. Structure 2019, 27, 566–578.
2. Monod, J.; Wyman, J.; Changeux, J. P. On the nature of allosteric transitions: A plausible model. J. Mol. Biol. 1965, 12, 88–118.
3. Koshland, D. E.; Némethy, G.; Filmer, D. Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry 1966, 5, 365–385.
4. Cooper, A.; Dryden, D. Allosteric without conformational change. Eur. Biophys. J. 1984, 11, 103–109.
5. Smock, R. G.; Giersch, L. M. Sending signals dynamically. Science 2009, 324, 198–203.
6. Petit, C. M.; Zhang, J.; Sapienza, P. J.; Fuentes, E. J.; Lee, A. L. Hidden dynamic alloster in a PDZ domain. Proc. Natl. Acad. Sci. U.S.A. 2009, 106, 18249–18254.
7. Tseng, S.-R.; Kalodimos, C. G. Protein activity regulation by conformational entropy. Nature 2012, 488, 236.
8. Saaavedra, H. G.; Wrabl, J. O.; Anderson, J. A.; Li, J.; Hilser, V. J. Dynamic alloster can drive cold adaptation in enzymes. Nature 2018, 558, 324.
9. Ahuja, L. G.; Aoto, P. C.; Kornev, A. P.; Veglia, G.; Taylor, S. S. Dynamic alloster-based molecular workings of kinase: peptide complexes. Proc. Natl. Acad. Sci. U.S.A. 2019, 116, 15052–15061.
10. Banerjee-Ghosh, K.; Ghosh, S.; Mazal, H.; Riven, I.; Haran, G.; Naaman, R. Long-Range Charge Reorganization as an Allosteric Control Signal in Proteins. J. Am. Chem. Soc. 2020, 142, 20456–20462.
11. Fuentes, E. J.; Der, C. J.; Lee, A. L. Ligand-dependent dynamics and intramolecular signaling in a PDZ domain. J. Mol. Biol. 2004, 335, 1105–1115.
12. Fuentes, E. J.; Gilmore, S. A.; Mauldin, R. V.; Lee, A. L. Evaluation of energetic and dynamic coupling networks in a PDZ domain protein. J. Mol. Biol. 2006, 364, 337–351.
13. Kumawat, A.; Chakrabarty, S. Hidden electrostatic basis of dynamic alloster in a PDZ domain. Proc. Natl. Acad. Sci. U.S.A. 2017, 114, E5825–E5834.
14. Kumawat, A.; Chakrabarty, S. Protonation-Induced Dynamic Allostery in PDZ Domain: Evidence of Perturbation-Independent Universal Response Network. J. Phys. Chem. Lett. 2020, 11, 9026–9031.
15. Gautier, C.; Laursen, L.; Jemth, P.; Gianni, S. Seeking allosteric networks in PDZ domains. Protein Eng. Des. Sel. 2018, 31, 367–373.
16. Thayer, K. M.; Lakhani, B.; Beveridge, D. L. Molecular dynamics–Markov state model of protein ligand binding and Allostery in CRIB-PDZ: conformational selection and induced fit. J. Phys. Chem. B. 2017, 121, 5509–5514.
17. Doyle, D. A.; Lee, A.; Lewis, J.; Kim, E.; Sheng, M.; MacKinnon, R. Crystal structures of a complexed and peptide-free membrane protein–binding domain: molecular basis of peptide recognition by PDZ. Cell 1996, 85, 1067–1076.
18. Cabral, J. H. M.; Petosa, C.; Sutcliffe, M. J.; Raza, S.; Byron, O.; Poy, F.; Marfatia, S. M.; Chishti, A. H.; Lid-
(19) Ballif, B. A.; Carey, G. R.; Sunyaev, S. R.; Gygi, S. P. Large-scale identification and evolution indexing of tyrosine phosphorylation sites from murine brain. J. Proteome Res. 2007, 7, 311–318.

(20) Zhang, J.; Petit, C. M.; King, D. S.; Lee, A. L. Phosphorylation of a PDZ domain extension modulates binding affinity and interdomain interactions in postsynaptic density-95 (PSD-95) protein, a membrane-associated guanylate kinase (MAGUK). J. Biol. 2011, 286, 41776–41785.

(21) Bozovic, O.; Jankovic, B.; Hamm, P. Sensing the allosteric force. Nat. Commun. 2020, 11, 5841.

(22) Bozovic, O.; Zanobini, C.; Gulzar, A.; Jankovic, B.; Buhrke, D.; Post, M.; Wolf, S.; Stock, G.; Hamm, P. Real-time observation of ligand-induced allosteric transitions in a PDZ domain. Proc. Natl. Acad. Sci. USA 2020, 117, 26031–26039.

(23) Jankovic, B.; Gulzar, A.; Zanobini, C.; Bozovic, O.; Wolf, S.; Stock, G.; Hamm, P. Photocontrolling protein–peptide interactions: From minimal perturbation to complete unbinding. J. Am. Chem. Soc. 2019, 141, 10702–10710.

(24) Flint, D. G.; Kumita, J. R.; Smart, O. S.; Woolley, G. A. Using an azobenzene cross-linker to either increase or decrease peptide helix content upon trans-to-cis photoisomerization. Chem. Biol. 2002, 9, 391–397.

(25) Lörenz-Fonfría, V. A.; Kandori, H. Transformation of time-resolved spectra to lifetime-resolved spectra by maximum entropy inversion of the Laplace transform. Appl. Spectrosc. 2006, 60, 407–417.

(26) Buhrke, D.; Oppelt, K. T.; Heckmeier, P. J.; Fernandez-Teran, R.; Hamm, P. Nanosecond protein dynamics in a red/green Cyanobacteriochrome revealed by transient IR spectroscopy. J. Chem. Phys. 2020, 153, 245101.

(27) Nägele, T.; Hoche, R.; Zinth, W.; Wachtveitl, J. Femtosecond photoisomerization of cis-azoazobenzene. Chem. Phys. Lett. 1997, 272, 489–495.

(28) Spörlein, S.; Carstens, H.; Satzger, H.; Renner, C.; Behrendt, R.; Moroder, L.; Tavan, P.; Zinth, W.; Wachtveitl, J. Ultrafast spectroscopy reveals sub-nanoscale peptide conformational dynamics and validates molecular dynamics simulation. Proc. Natl. Acad. Sci. USA 2002, 99, 7998–8002.

(29) Ihalainen, J. A.; Bredenbeck, J.; Pfister, R.; Helbing, J.; Chi, L.; van Stokkum, I. H. M.; Woolley, G. A.; Hamm, P. Folding and unfolding of a photoswitchable peptide from picoseconds to microseconds. Proc. Natl. Acad. Sci. U.S.A. 2007, 104, 5383–5388.

(30) Barth, A.; Zacherp, C. What vibrations tell us about proteins. Q. Rev. Biophys. 2002, 35, 369–430.

(31) Stock, G.; Hamm, P. A non-equilibrium approach to allosteric communication. Philos. Trans. R. Soc. Lond. B Biol. Sci. 2018, 373, 20170187.

(32) Bowman, G. R.; Pande, V. S.; Noe, F. An introduction to Markov state models; Springer: Heidelberg, 2013.

(33) Sengupta, U.; Strödel, B. Markov models for the elucidation of allosteric regulation. Phil. Trans. R. Soc. B 2018, 373, 20170178.

(34) Swain, J. F.; Gierasch, L. M. The changing landscape of protein allostery. Curr. Opin. Struct. Biol. 2006, 16, 102–108.

(35) Tsai, C. J.; del Sol, A.; Nussinov, R. Allostery: Absence of a Change in Shape Does Not Imply that Allostery Is Not at Play. J. Mol. Biol. 2008, 378, 1–11.

(36) Tsai, C. J.; Nussinov, R. A unified view of "how allostery works". PLoS Comput. Biol. 2014, 10, e1003394.

(37) Hilser, V. J.; Wrabl, J. O.; Motlagh, H. N. Structural and energetic basis of allostery. Annu. Rev. Biophys. 2012, 41, 585–609.

(38) Gianni, S.; Engström, Å.; Larsson, M.; Calosci, N.; Malatesta, E.; Eklund, L.; Nang, C. C.; Travaglini-Alcatalegl, C.; Jemth, P. The kinetics of PDZ domain-ligand interactions and implications for the binding mechanism. J. Biol. Chem. 2005, 280, 34805–34812.

(39) Schottle, F.; Soman, J.; Olson, J. S.; Wulff, M.; Auffrads, P. A. Picosecond time-resolved X-ray crystallography: probing protein function in real time. J. Struct. Biol. 2004, 147, 235–246.

(40) Knapp, J. E.; Pahl, R.; Šrajer, V.; Royer, W. E. Allosteric action in real time: Time-resolved crystallographic studies of a cooperative dimeric hemoglobin. Proc. Natl. Acad. Sci. U.S.A. 2006, 103, 7649–7654.

(41) Cammarata, M.; Levantino, M.; Wulff, M.; Cupane, A. Unveiling the timescale of the R-T transition in human hemoglobin. J. Mol. Biol. 2010, 400, 951–962.

(42) Shibayama, N. Allosteric transitions in hemoglobin revisited. Biochim. Biophys. Acta Gen. Subj. 2020, 1864, 129335.

(43) Boyer, P. D. The ATP Synthase—A Splendid Molecular Machine. Ann. Rev. Biochem. 1997, 66, 717–749.

(44) Bredenbeck, J.; Helbing, J.; Hamm, P. Continuous scanning from picoseconds to microseconds in time resolved linear and nonlinear spectroscopy. Rev. Sci. Instrum. 2004, 75, 4462–4466.

(45) Hamm, P.; Kaindl, R. A.; Stenger, J. Noise suppression mechanism. J. Biol. Chem. 2005, 280, 34805–34812.

(46) Feng, Y.; Vinogradov, I.; Ge, N.-H. General noise suppression scheme with reference detection in heterodyne nonlinear spectroscopy. Opt. Express 2017, 25, 26262.