Dissecting time-resolved IR spectra of a Cyanobacteriochrome with kinetic labeling

David Buhrke*, Kerstin T. Oppelt, Philipp J. Heckmeier, Ricardo Fernandez-Tern and Peter Hamm
Department of Chemistry, University of Zürich, Zürich, Switzerland
* david.buhrke@chem.uzh.ch
(Dated: August 26, 2020)

Over the last decades, photoreceptive proteins were extensively studied with biophysical methods to gain a fundamental understanding of their working mechanisms and further guide the development of optogenetic tools from them. Time-resolved infrared (IR) spectroscopy is one of the key methods to access their functional non-equilibrium processes with high temporal resolution, but has the major drawback that experimental data is usually highly convoluted. Linking the spectral response to specific molecular events is a major obstacle and usually requires costly isotope labeling or mutagenesis methods. Here, we investigate a cyanobacteriochrome (CBCR) photoreceptor with a combined approach of transient absorption spectroscopy in the visible and IR wavelength regions. We obtain kinetic information in both spectral regions by frequency resolved lifetime analysis, in contrast to the widely used approach of global fitting. This technique allows us to analyze different regions in the IR response and compare them to kinetic labels obtained from the Vis data. We find that the non-equilibrium response differ when either the phycocyanobilin (PCB) cofactor or the protein backbone is chosen as an experimental observable. While spectroscopic signals associated with PCB evolve through well-separated intermediate states, a higher complexity in the amide I’ region indicates that changes in the protein structure can be better described by diffusion on a rugged energy landscape. We discuss the implications of these findings for sequential intermediate schemes and conclude on how the modeling of non-equilibrium dynamics depends on the point of view.

I. INTRODUCTION

Cyanobacteriochromes (CBCRs) are modular light-regulated enzymes that adjust the activity of catalytic output modules (e.g. a histidine kinase domain) through bistable photoswitching of one or more distant photosensory modules (PSMs). Thus, CBCRs enable cyanobacteria to regulate diverse cellular processes in response to changes in environmental light conditions. The PSMs adapt a GAF (cGMP-phosphodiesterase/adenylate cyclase/FhlA) fold motif and bind various bilin chromophores, such as phycocyanobilin (PCB), to one or more cysteine residues. CBCR PSMs are becoming increasingly popular as building blocks for optogenetic tools, because they can be fused to a variety of catalytic domains to allow for allosteric photocontrol of their respective reactions. To name a few examples, CBCRs recently have been engineered to function as adenyl cyclase[14] to control protein-protein interactions[8] and gene expression in bacteria[14]. Furthermore, the small size, spectral diversity and fluorescence properties of CBCR PSMs are promising for applications as markers in fluorescence multiplexing and super-resolution microscopy[9].

Most of the considered biotechnological applications introduce directed modifications of the respective PSMs, and thus detailed knowledge about the PSM structure and dynamics on a molecular level is required. While X-ray crystallography[10,11] and NMR spectroscopy[12] provide structural information for thermally stable (parent) states of the CBCRs, transient spectroscopies are valuable tools to study the photo-induced non-equilibrium dynamics[13,14].

Here, we investigate a PSM of the CBCR Slr1393 from Synechocystis PCC6803 by transient absorption spectroscopy in the infrared (IR) and visible (Vis) spectral regions. This protein domain binds PCB and is located in a sequence after two non-photoactive GAF domains (counted from the N-terminus), therefore denoted Slr-g3 in the following. Slr-g3 naturally acts as a light-regulated histidine kinase, but by artificially fusing Slr-g3 to an adenyl cyclase, photocontrol over this domain was also achieved[15]. Slr-g3 is a small protein domain (190 aa, 22 kDa) that converts reversibly between a red-light absorbing (Pr) and a green-light absorbing (Pg) parent state[16]. The crystal structures in both parent states were recently solved (Fig. 1A and B) and display distinct structural differences of PCB and the protein backbone. In the Pr state, PCB is found in the 15Z configuration (Fig. 1C), closely resembling the Pr state of phytochromes. In the Pg state on the other hand, one methine bridge is isomerized (15E), and the outer pyrrole rings A and D are twisted out of plane, leading to an effective reduction in conjugation length and thus the characteristic hypsochromic shift of the absorption maximum (Fig. 1D). The Pg and Pr states differ also with respect to the protein structure, e.g. the position of a tryptophan residue and α-helicity (highlighted in Fig. 1F). The differences in the chromophore and protein configuration in Pr and Pg are reflected in changes of the Vis and IR absorption spectra (Fig. 1D and E). Similar to other photoreceptor proteins, the light-induced reactions of Slr-g3 can be described by a photocycle scheme (Fig. 1F), where electronically excited states decay via a series of ground state intermediates (GSIs) to the respective product states. We employ transient Vis and IR spectroscopy in parallel to study the photocycle reactions in a time window between 100 ps and 42 ps. Here, the kinetics obtained from Vis measurements provide a selective labeling of the PCB chromophore’s response, and thus aid the interpretation of the complex IR signals. With this
kinetic labeling approach, we are able to provide mechanistic insights about the coupling of protein and chromophore rearrangements.

II. MATERIALS AND METHODS

Expression and Purification

Slr-g3 was expressed and assembled with PCB in *Escherichia coli* BL21 cells in darkness. The holo-Slr-g3 expressing cell line was a generous gift from the lab of Thomas Friedrich (TU Berlin). The protein was purified under native conditions via Ni-affinity chromatography and a His6-Tag N-terminal to the Slr-g3 domain and desalted using a Sephadex HiPrep 26/10 column (GE Healthcare Bio-Sciences, Uppsala, Sweden) into a final buffer containing 50 mM Tris (pH 7.8 or 7.4 for subsequent D₂O exchange), 300 mM NaCl and 5 mM EDTA. For all IR experiments, the samples were prepared in D₂O buffer using the following protocol: samples were lyophilized, re-dissolved in D₂O and kept in the dark at 4°C for at least 5 h to ensure complete H/D exchange. The samples were lyophilized again for storage and dissolved in D₂O only immediately before the measurements. The integrity of the samples during the lyophilization steps was monitored by recording UV-Vis spectra of the Pr and Pg states before and after the procedure and no differences were found (Fig. S1).

Steady-state spectroscopy

Steady-state UV-Vis spectra were recorded with a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a UV-2450 UV-Vis Spectrophotometer (Shimadzu, Nakagyo-ku, Kyoto, Japan). The samples were prepared either in the Pr or Pg states by illumination with green and red LED arrays (LIU630A and LIU525B, Thorlabs, Newton, MA, USA) as starting points for all experiments. FTIR difference spectra were recorded with a Tensor 27 spectrometer (Bruker, Ettlingen, Germany).

Transient absorption spectroscopy

In all time-resolved Vis and IR experiments the samples were prepared under the same conditions to ensure maximum comparability of the datasets. The sample with a concentration of 0.7 mM (computed with the ExPASy ProtParam tool from the sequence and OD₂₈₀(0.1 mm)= 0.25) was cycled in a closed system with a peristaltic pump to ensure sufficient sample exchange in the probe spot. This closed system included a reservoir where the sample was illuminated with the same LED arrays that were used in the static experiments to prepare either the Pr or Pg state and was constantly purged with N₂. The measurement cell consisted of two 2-mm thick CaF₂ windows separated by a 50 μm Teflon spacer. Transient Vis and IR experiments employed a pump-probe scheme with two electronically-synchronized Ti:Sapphire laser systems running at 2.5 kHz. Pump-probe difference spectra were obtained by alternately blocking consecutive pump laser shots using a mechanical chopper and acquired up to the maximum delay value of 42 μs with the same delay times. Spectral traces at negative delay times (-10 ns) were used for background subtraction. The time resolution was limited by the length of the pump pulse (60 ps, while the synchronization jitter of the setup was 10 ps). The linear polarization of the pump pulse was set to an angle of 54.7° relative to the p-polarized probe pulse (magic angle). A multichannel referencing scheme was used to suppress noise in all experiments.

For the UV pump pulses, the respective laser was tuned to 760 nm, such that second harmonic generation in a BBO crystal produced pulses with a center wavelength...
of 380 nm (see Fig. 1D). The compressor stage of the amplifier was bypassed and stretched pulses of ca. 60 ps FWHM duration (determined on the rising absorption edge on a silicon wafer) and a power of 1 µJ were employed to ensure mild pumping conditions and minimize sample degradation. The visible probe pulses were generated by tightly focusing ca. 1 µJ of the 800 nm pulses generated by the probe laser into a stationary 3 mm thick sapphire plate. After passing the sample, the probe beam was collimated, dispersed by a UV transmission grating (Thorlabs, 830 mm⁻¹), focused by 75 mm fused silica lenses onto a 2048-pixel CMOS line array (Synertronic Designs). The probe spectral axis was calibrated by fitting the position of the transmission maxima of several interference filters, and the light intensity was controlled by using broadband neutral density filters to prevent saturation of the detector. Color-balancing filters were used to homogenize the light intensity profile of the probe and reference beams, and to filter out stray pump light. The obtained spectral resolution was ca. 0.8 nm after binning of four adjacent pixels to improve the signal-to-noise ratio, yielding 512 effective pixels.

Mid-IR probe pulses centered at 1600 or 1720 cm⁻¹ (duration ca. 100 fs) were generated in an optical parametric amplifier (OPA) passed through a spectrograph and detected in a 2x64 MCT array detector with a spectral resolution of ≈ 2 cm⁻¹/pixel. The two spectral regions had an overlap of ca. 20 cm⁻¹ which was used to join the spectral regions and obtain the representations spanning the range from 1520 to 1780 cm⁻¹. An FTIR spectrum of water vapor and the water vapor lines of the non-purged setup were used for frequency calibration.

RESULTS AND DISCUSSION

Transient Vis absorption

The largest signals in the Pr → Pg transition found at early times (labeled I in Fig. 2A,D) are associated with the excited electronic state (Pr*). This time window has been studied previously with high time resolution and the signals were assigned to excited state absorption (ESA, mainly <550 nm), stimulated emission and ground state bleach (SE and GSB, strong negative band around 620-750 nm). The region in between (550-620 nm) contains overlapping contributions from ESA and GSB. Similar excited-state spectra were also obtained for several related red/green CBCRs from Nostoc punctiforme, albeit with large variations in lifetimes. In the case of Slr-g3, the excited state vanishes after approximately 1 ns, and the remaining signals, which are roughly a factor of 10 smaller, can be safely assigned to ground state photoproducts. Up to approximately 1 µs, ground state dynamics are observed, mainly characterized by a successive blue-shift of the photoproduct absorption in the investigated time window (Fig 2C). We find no notable differences between the spectral traces at 1 µs and the last transient at 42 µs, indicating a meta-stable configuration that prevails during this time window. Comparison of the last transient (42 µs, Fig. 2C) to the steady-state Vis ”Pg-minus-Pr” difference spectrum (Fig. 2B) indicates that Pg is not yet formed within the time frame of the experiment, thus this signal must correspond to a ground state intermediate rather than the Pg state. This result is in good agreement with flash photolysis experiments of Slr-g3, which showed a similar trace with a broad maximum around 570 nm in the early microsecond range, while the formation of Pg proceeds with a much longer time constant of 1.1 ms.

In the reverse direction (Pg → Pr), the earliest signals from the Pg* state (Fig. 2H) are also in good agreement with the literature on Slr-g3 and related red/green CBCRs. Here, the positive signal around 700 nm was assigned to ESA, while the negative features at 525 and 625 nm correspond to GSB and SE, respectively. Compared to Pr*, Pg* decays faster and more productively, as judged from the relative intensities of the excited- and ground state related bands (compare different scales in Fig. 2C and G, D and H). The literature values for photo-chemical quantum yields are 0.08 (Pg formation) and 0.3 (Pr formation), respectively. The ground state dynamics appear more complex than in the Pr → Pg transition. Notably, a local maximum of the photoproduction absorption is found at 600 nm and around 10 ns, before it decays and red-shifts at later times (III), while the GSB band stays nearly constant over the observed time window. The dynamics on this timescale are evidence for intermediate states that were not previously reported. Pr formation is not finished within the investigated time window and proceeds within 1 ms from an orange-absorbing intermediate.

Transient IR absorption

The transient IR and Vis experiments were conducted under the same experimental conditions to ensure maximal comparability (see materials and methods for details), and Fig. 3 shows the IR data in the same representation as the Vis results in Fig. 2. IR spectra in the region between 1520 and 1780 cm⁻¹ are typically dominated by contributions from the PCB chromophore (C=C, C=N and C=O stretching modes) superimposed with amide I' signals originating from structural changes in the protein backbone. Furthermore, C=O stretching vibrations from carboxylic groups also appear in this region. Due to the strong overlap of these different contributions, precise assignment of signals to specific vibrational modes is generally difficult, even with the aid of isotope labeling experiments or calculations.

This said, useful information may be extracted with great care to avoid overinterpretation. First, the steady-state IR difference spectra (Fig. 3B,F) closely resemble the results obtained for the highly homologous red/green CBCR AnPixJ-g2 from Anabaena PCC 7120. This related GAF domain was investigated by FTIR spectroscopy with a uniformly 13C,15N-labeled PCB chromophore and thus some bands were assigned to PCB or...
the protein environment on this basis. For example, the difference signal at 1690/1700 cm\(^{-1}\) (IV) was assigned to the PCB D-ring carbonyl stretch. This finding is consistent with the present results: GSB signals at the respective frequencies are found in the traces of Pg* and Pr* in the picosecond regime (Fig. 3 D,H), originating from the isomerization reaction and rotation of pyrrole ring D.

Second, the strongest negative band in both excited state signals (Pr* and Pg*) is observed around 1610 cm\(^{-1}\) (V), similar to the dominant feature in the excited states of other PCB-binding photoreceptors such as the cyanobacterial phytochrome Cph\(^{17-19}\) or the CBCRs Thr092\(^{20,21}\) and TePix\(^{22}\). This ubiquitous feature is commonly assigned to the bleach of PCB C=C stretching signals upon electronic excitation. Recent resonance Raman experiments of Slr-g3 in conjunction with QM/MM normal mode calculations predict high IR intensities for a normal mode dominated by C=C stretching motions mainly at the B and C pyrrole rings at this frequency, which validates the assignment reference to Bulurke2020. This strong feature overlaps only weakly with the amide I' region and hence serves as a marker for the conjugated system of PCB in the transient IR data.

Third, the time evolution of the ground-state signals allows an assignment of spectral features. It was recently shown that the Vis signals are selective and sensitive probes of the effective conjugation length in PCB in the case of Slr-g3\(^{23}\), while IR spectra are expected to contain contributions from both PCB and protein. Thus, events that show up strongly in the Vis data likely originate from adjustments of dihedral angles between the pyrrole rings, which have a large impact on the effective conjugation length but only affect IR signals that are also associated with the chromophore, e.g. the C=C stretching in the region around 1610 cm\(^{-1}\). If features associated with certain time scales appear dominant in IR transients in the amide I region with only small counterparts in the Vis, they likely originate from structural changes in the protein surroundings and have only minor impact on the conjugated system of PCB. We refer to this idea as kinetic labeling, because it allows to separate events that dominantly affect the chromophore or the protein by comparing the kinetics at certain frequencies in the IR to the Vis data. Just like chemical labeling uses specific functional groups or isotopes to allow for an assignment of spectral features, the time scales (kinetic labels) obtained from the Vis spectra allow the assignment of IR signals to the conjugated system of the PCB chromophore. This approach requires a frequency resolved kinetic analysis of the IR data.

**Lifetime analysis**

All datasets were fit to multieponential functions\(^{37-40}\)

\[
S(\omega, t) = a_0(\omega_i) - \sum_i a(\omega_i, \tau_j)e^{-t/\tau_j},
\]

where the index \(j\) refers to a kinetic component with time constant \(\tau_j\), and \(i\) to a probe frequency \(\omega_i\). The time constants \(\tau_j\) were xed and distributed equidistantly on a logarithmic scale with 10 terms per decade, while the amplitudes \(a(\omega_i, \tau_j)\) were the free tting parameters. A penalty function was added to the RMSD of the \(t, \max\) for processes with similar time-scales are involved. Both approaches are potentially unstable, in particular if processes with similar time-scales are involved. In the case of the lifetime analysis discussed here, two different regularisation criteria were tested to stabilize the fits\(^{11}\). Here, the discrepancy criterion yielded reasonable lifetime spectra, while the alternative approach of regularisation with the L-curve criterion resulted in overfitting of the data (included in the SI).

The average amplitude over all frequencies is termed the dynamical content\(^{12}\) \(D(\tau_j)\), which quantifies the overall dynamics at a certain time point \(j\):
compared to the excited state, Fig. 2 and 3 A,C). Albeit low photochemical quantum yield (10-20x smaller signals both Vis and IR data display only weak signals due to the of subsequent ground state heat signals. At later times, process in the IR is most likely caused by the contribution IR data (Fig. 4A-D). The apparent slower kinetics of this (associated with the decay of the electronic excited state the Vis and IR regions.

In the case of tetrapyrrole photoreceptors) and start our

for ground state intermediates (typically Lumi and Meta decided to dispense with any established naming scheme

determined at the same delay times. Roman numerals IV-V refer to details discussed in the text.

Lifetime spectra

To provide minimally biased data analysis, we decided to dispense with any established naming scheme for ground state intermediates (typically Lumi and Meta in the case of tetrapyrrole photoreceptors) and start our analysis by comparing time scales that are obtained in the Vis and IR regions.

In the Pr→Pg transition, the overall maximum \(D(\tau_j)\) is associated with the decay of the electronic excited state (VI), observed at 600 ps in the Vis and 900 ps in the IR data (Fig. 4A-D). The apparent slower kinetics of this process in the IR is most likely caused by the contribution of subsequent ground state heat signals. At later times, both Vis and IR data display only weak signals due to the low photochemical quantum yield (10-20x smaller signals compared to the excited state, Fig. 2 and 3 A,C). Albeit small, these signals clearly show an evolution within the investigated time window and thus at least one dominant lifetime is expected in the fits. Scaling \(D(\tau_j)\) by a factor of 10x reveals amplitude maxima in the lifetime spectra at 25 and 100 ns (VII, Fig. 4B,D). Due to the discrepancy in the extracted lifetimes that originates from the low S/N ratio, we cannot conclude whether they correspond to the same process or not. This imprecise kinetic information derived from small signals (ca. 10 µOD in the IR) demonstrates the limitations of this method and allows us to assess the quality of the other fits.

In the reverse direction (Pg→Pr, Fig. 4E-H), the excited-state decay also constitutes the dominant feature in the lifetime spectra, with maxima at 150 ps in the Vis data and 300 ps in the IR (VIII). At later times, stronger amplitudes at different time scales are observed in both datasets compared to the top panels, indicating that in this switching direction, more local energy minima are populated. A closer inspection of the lifetime spectra reveals connections between the Vis and IR datasets which can be used to dissect the superimposed information in the IR spectra, indicated by dashed horizontal lines in Fig. 4

**Kinetic labeling**

To understand the kinetic labeling approach, consider the following. In all experiments, samples were prepared in either the Pr or Pg states by background illumination with red and green LED arrays. While the Pr state is populated close to 100 % in this way, illumination with a red light source leads to a photostationary equilibrium that contains a majority of Pg, but also a considerable amount of Pr. By visual inspection of the static Vis spectra, we estimate ca. 10 % residual Pr in the Pg samples (Fig. 1D). When these samples are excited at 380 nm, the residual Pr contribution is also excited and thus the Pr signals contribute to the Pg response. We chose not to subtract this contribution and demonstrate instead how it can be identified in the Pg spectra by exploiting the fact that Pg* decays faster then Pr* and thus carries a kinetic label. In both Pg→Pr lifetime spectra, the early times show two peaks that correspond to the decays of Pg* and Pr*. In the Vis dataset, these peaks in \(D(\tau_j)\) are clearly separated at 150 and 600 ps, while in the IR, the 900 ps decay of Pr* is detected in the form of a shoulder (Fig. 4E-H, VIII). Note that this separation of the Pr* related signals is not only true for the local maximum of \(D(\tau_j)\), but also positive and negative amplitudes are found in the same spectral regions in both the IR and Vis lifetime spectra at the respective time points.

Having demonstrated the applicability of kinetic labeling on the excited state signals, we turn to the subsequent evolution on the electronic ground state at times later than 1 ns. In the Pr→Pg reaction, the features in the lifetime spectrum are too small to yield reliable time constants for the process(es) observed in the nanosecond regime. On the other hand, this smallness also ensures that contribution to the Pg→Pr processes is negligible,

FIG. 3. Transient IR absorption data from 100 ps to 42 µs. A: Pr → Pg transition, red color code indicates positive absorbance change, blue negative change. B: steady state "Pg-minus-Pr" FTIR difference spectrum compared to selected spectral traces at 100 ps (D), 10 ns, 1 µs and 42 µs (C). E: Pg → Pr transition. The selected spectral traces in F-H were chosen at the same delay times. Roman numerals IV-V refer to details discussed in the text.
FIG. 4. Lifetime spectra and dynamical content $D(\tau_j)$ for all datasets. The color code is defined such that the decay of a positive (red) signal in the raw data is represented here by blue amplitude and vice versa. A: lifetime spectra calculated from the Pr → Pg Vis data plotted together with (B) the averaged dynamical content $D(\tau_j)$ (Eq. 2) for this dataset (black line). The same trace was scaled by a factor of x10 to reveal smaller features (blue line). Dashed horizontal lines are intended to guide the eye towards events that appear in both datasets. C,D: lifetime spectrum and $D(\tau_j)$ of Pr → Pg IR data. E,F: Pg → Pr Vis data. G,H: lifetime spectrum of Pg → Pr IR data. Roman numerals VII-XII refer to details discussed in the text.

unlike the decay of Pr$^*$. In the Pg→Pr direction, the second maximum of $D(\tau_j)$ after the decay of Pg$^*$ is detected around 40 ns (Fig. [G,H, IX]). Here, strong amplitudes appear in the amide I' region between 1620 and 1680 cm$^{-1}$, and a small feature is observed at 1600 cm$^{-1}$ slightly earlier at 10 ns. In the Vis lifetime spectrum, a corresponding weak signal is also detected at 10 ns (Fig. [E,F, X]). We interpret this pattern as follows: on the 40 ns time scale, the protein backbone undergoes somewhat larger structural rearrangements that lead to the large signals in the amide I' region. These are coupled to or eventually triggered by earlier minor changes in the chromophore geometry, reflected by the weak signals in the Vis, and in the IR marker band of PCB at 1600 cm$^{-1}$. Here, the frequency resolution of the lifetime fit allows to separate the kinetic component at 1600 cm$^{-1}$ and 10 ns from the strong amide I' transition at 40 ns. Thus, the 1600 cm$^{-1}$ component can be directly related to the Vis lifetime spectrum.

The Vis lifetime spectrum is dominated by two features at 800 ns (XI) and 10 µs (XII), both of which are mirrored by features with similar negative and positive amplitudes around 1600 cm$^{-1}$ in the IR (Fig. [G,H]). Note that blue-shifts in the Vis (at 10 and 800 ns) are accompanied by upshifts of the C=C stretching mode as a consequence of a shortening conjugated system. Consequently, the red shift at 10 µs is accompanied by a down-shift in the IR and indicates a conjugation elongation. The effective conjugation length of PCB changes on these time scales most likely due to twisting of the outer pyrrole ring$^{23,24}$. The two clearly separated maxima of dynamical content (XI and XII) imply the existence of at least two energy barriers, corresponding to at least three meta-stable ground state intermediates with different discrete chromophore geometries that interconvert on this time scale. In the amide I' region, on the other hand, peaks are not clearly separated and also have strong amplitudes between 800 ns and 10 µs. This indicates a protein response to these discrete changes in chromophore geometry happening on the same time scales, albeit rather stretched out continu-
ously in time. These findings are in a striking contrast to Rhodopsin, where the same fitting procedure applied to step-scan IR data yielded the similar amplitude maxima across the entire region from 1000-1800 cm$^{-1}$, associated with two discrete changes between the Lumi, Meta-I and Meta-II GSI.

**Photocycle Models**

Since the early 1970s, many different photoreceptor proteins were studied in detail, often with the goal to construct photocycle models involving the interconversions of discrete GSIs. These schemes and nomenclature often had to be adjusted after new experimental results obtained with different techniques were available. In the case of tetraropyrrole photoreceptors, the common nomenclature was derived from cryo-trap Vis absorption experiments on *Avena sativa* phy A and denotes the primary ground-state photoproducts as "Lumi" and the subsequent thermal intermediates as "Meta" states. This naming scheme was extended several times when new experimental results were available (e.g. early/late Lumi or several cases of Meta sub-states).

A common approach that allows to adopt this nomenclature to CBCRs with their diverse photocycles is to fit time-resolved data with multiple exponentials and then infer a photocycle model from the obtained time constants and decay-associated spectra. However, regardless of how kinetic information has been determined, by frequency-resolved lifetime analysis or by multi-exponential fitting, there is no unique way to assign these kinetic constants to a molecular model, as the problem is inherently underdetermined. To see why that is so, consider a kinetic matrix of a system with $n$ states, which has $n \times (n-1)$ independent matrix elements, but only $n-1$ non-zero eigenvalues. The eigenvalues are directly related to the observed timescales, but that information is not sufficient to determine all $n \times (n-1)$ elements of the kinetic matrix. In an analysis of so-called decay-associated spectra, or the like, one therefore assumes a sequential model, ordering timescales from fast to slow, which renders the assignment unique.

A frequency-resolved lifetime analysis was used in a previous study of the excited-state dynamics of SIR-g3, and yielded essentially the same information content as kinetic modeling with a sequential scheme of intermediate states to obtain evolution-associated difference spectra in the early ns regime. In this report, we reproduce these lifetime spectra in the Vis region up to 1 ns, but we extend this approach to longer times and the IR where the protein with its intrinsic ruggedness takes over the control of the kinetics. This becomes evident when Figures 4 E and G are compared. The Vis data and the chromophore marker band at 1600 cm$^{-1}$ appear like a series of well-separated intermediate states (Fig. 4E,F). However, the amide I' response in the same time window is much less discrete and spread out in time. Here, the assumption of sequential intermediate states is very likely to break down. Instead, we propose to think of the kinetics in this regime in terms of a Markov state model with a set of local minima on the rugged free energy surface of the protein, many of which are kinetically connected in the sense of a network. What we observe are the implied timescales of that Markov state model. These transitions include small structural changes associated with small energy barriers and small spectroscopic features, which constitutes a problem for a nomenclature involving discrete GSIs. That approach needs a threshold be set in order to define which changes are big enough to be considered as a new (sub-)GSI.

**III. CONCLUSIONS**

Altogether, we show that by employing a frequency resolved instead of global lifetime analysis, time scales of the chromophore and protein structural rearrangements can be identified. These are coupled to one another and occur on similar time scales, but do not necessarily share the same time constants and discreteness. Especially, the complex lifetime spectra in the amide I' region (Fig. 4G), indicate that it would be an oversimplification to describe the protein response by a scheme of successive discrete intermediates. Thus, we propose to understand this system in terms of a mutual interplay of coupled structural rearrangements at different sites. While the PCB chromophore undergoes a series of rather discrete transitions, the protein environment explores more conformational degrees of freedom as it diffuses on a rugged energy landscape, which can potentially be described by a Markov state model. Starting from this point, the different sites of the protein may be addressed independently in future studies to analyse their contributions to the dynamic amide I' signals.

**A. Acknowledgements**

The authors thank Thomas Friedrich and co-workers for providing the SIR-g3 expressing *E.coli* cell line, Olga Bozovic for help with protein expression, Roland Zehnder and Jan Helbing for technical support and Friedrich Siebert and Chavdar Slavov for helpful comments. The work has been supported by the Swiss National Science Foundation (SNF) through the NCCR MUST and Grant 200020B_188694/1.

---

1. N. C. Rockwell and J. C. Lagarias, ChemPhysChem 11, 1172 (2010).
2. N. C. Rockwell and J. C. Lagarias, Curr. Opin. Plant Biol. 37, 87 (2017).
