Overcoming the Challenges of Measuring the Irreversible Photochemical Dynamics of a 'Tricky' Protein using Time-Resolved Infrared Spectroscopy: the B_{12}-Dependent Transcriptional Regulator, CarH

Contact *alex.jones@npl.co.uk*

I. S. Camacho
Biometrology, Chemical and Biological Sciences
National Physical Laboratory, Teddington, Middlesex, UK

I. V. Sazanovich
Central Laser Facility
Rutherford Appleton Laboratory, Didcot, UK

E. Gozzard
Central Laser Facility
Rutherford Appleton Laboratory, Didcot, UK

M. Towrie
Central Laser Facility
Rutherford Appleton Laboratory, Didcot, UK

N. T. Hunt
Department of Chemistry and York Biomedical Research Institute, University of York, York, UK

A. R. Jones
Biometrology, Chemical and Biological Sciences
National Physical Laboratory, Teddington, Middlesex, UK

Introduction

Throughout nature, organisms respond to the changing ambient light conditions through the action of both visual and non-visual photoreceptor proteins. Although it has been known for decades that derivatives of vitamin B_{12} are sensitive to light,[1] it was only recently discovered that B_{12} has a role as photoreceptor chromophore.[2] In the dark, the bacterial transcriptional regulator, CarH, binds to coenzyme B_{12} (5'-deoxyadenolsylcobalamin, AdoCbl), which triggers tetramer formation and binding to DNA, thus blocking transcription. Photolysis of the upper axial ligand of AdoCbl results in structural changes in the protein that lead to tetramer dissociation and DNA release.[3, 4] Thus, light switches on the genes that control the biosynthesis of carotenoids in response to photooxidative stress. This switching mechanism is now being exploited as the basis of engineered tools that enable optical control of neuronal activity,[5] hydrogels,[6, 7] transgene expression[8] and cell adhesion.[9]

In 2015, we proposed a photochemical mechanism for CarH – the first for any B_{12}-dependent photoreceptor – over femtoseconds-seconds using data from UV-visible transient absorption (TA) spectroscopy.[4] These data revealed that the photochemistry of AdoCbl when bound to CarH is quite different to when it is free in solution, and gave tantalizing clues about structural changes in the protein environment around the chromophore. Questions remain, however, about how the protein tunes the AdoCbl photochemistry and how the light signal is transduced through the protein to result in disassembly of the protein complex. The time-resolved infrared (TRIR) capability at the CLF Ultra facility is ideally suited to help probe the interactions between chromophore and protein. This not only includes the sub-nanosecond vibrational changes that occur upon photoexcitation of the chromophore and their coupling to the protein, but also the protein structural changes that result from photoexcitation on longer timescales (microsecond – milliseconds). Data are collected in a region of the mid-IR (1500-1700 cm^{-1}) where both B_{12} derivatives[10] and their dependent proteins[11] yield significant signals.

Yet there are numerous challenges to TRIR measurements with CarH. These are mostly rooted in the fact that TRIR requires high sample concentrations and that the CarH photoconversion is irreversible. Like many proteins, a limited amount of CarH is available, making sample efficiency a key consideration. To exacerbate this, the irreversibly formed CarH photoproduct (a monomer) is less soluble than the dark state tetramer, and thus precipitates during data acquisition at the high concentrations needed for TRIR, resulting in light scattering. AdoCbl is also highly sensitive to ambient light < 600 nm, which means CarH must be handled under far red light to stop unwanted (and irreversible) photoconversion and therefore loss of sample.

Here, we describe optimized methods for CarH sample preparation and handling and for TRIR data acquisition that successfully overcome these challenges. Our approach yields reliable and high-quality data and should be adaptable for the measurement of other 'tricky' proteins by TRIR.

Experimental

CarH does not express with AdoCbl bound. It is therefore necessary to incorporate the chromophore into the protein and for stability reasons this must be done immediately prior to measurement. Briefly, a solution of the CarH apo-protein (< 150 µM to remain soluble) is incubated with a 5-fold excess of AdoCbl for ≥10 mins. Unbound AdoCbl is then removed using a size-exclusion column, which results in a 1.5-fold dilution. The holo-CarH is then brought up to working concentration (200-300 µM) using centrifugal filter devices. The entire process is conducted under red light, takes several hours and a manageable protocol produces only ~ 2 mL of working solution.

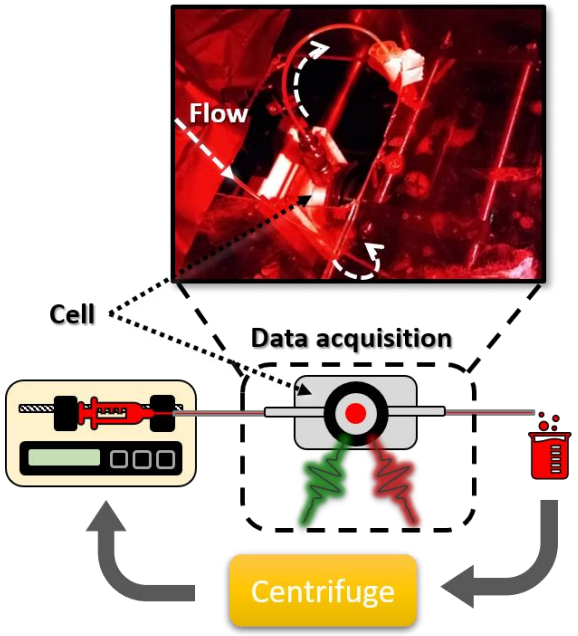

Figure 1. *Schematic of the final experimental setup (bottom) where the sample (red) flows (left to right) from a syringe pump, via tubing, through the modified cell to waste. The 'waste' is then centrifuged to remove photoconverted precipitate and the supernatant recycled for further data acquisition. Visible pump (green) and midIR probe (red) laser light are shown incident to the cell window. A photograph (top) shows the flow set up* in situ *under red ambient light. The direction of sample flow from the pump (out of shot) is indicated using white dashed arrows.*

In our previous laser spectroscopy studies with B_{12} species and CarH, data were acquired with the sample either flowed cyclically through the optical cell (TRIR)[10, 11] or with the sample stirred throughout using a magnetic bead (TA).[4] Any signal from the photoproduct was either minimal and/or could be accounted for during data analysis. The UV-vis TA used a pathlength (2 mm) that allowed CarH concentrations that are below the point at which the photoconverted monomer precipitates (< 150 µM). Unfortunately, because of the short optical pathlengths required for TRIR (in this case 100 µm), we could only achieve good signal with concentrations of ~200-300 µM. Using cyclical flow and a peristaltic pump as previously,[10, 11] it was not possible to avoid the accumulation of precipitate within the optical window and associated light scattering that obscures the signal. It was therefore necessary to use one-way flow from a syringe pump, through a modified (for details see below), small Harrick cell to waste (*Fig. 1*). Typical experimental parameters were 10 scans per measurement, an averaging time of 7.5 s, and a 525 nm excitation pump laser with a pulse energy of 1 µJ.

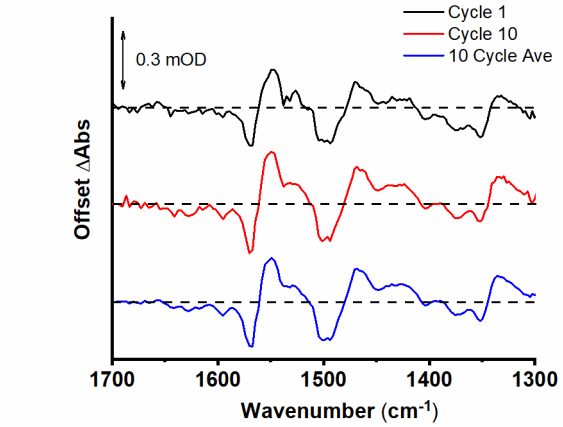

Figure 2. *TRIR difference spectra at 2 ps following photoexcitation of AdoCbl at 525 nm in aqueous buffer. The data illustrate the scan-to-scan reproducibility of the acquisition method in Fig. 1. Plots are offset for clarity; dashed lines indicate zero spectral amplitude; and a scale bar is indicated.*

Results and Discussion

The limited working sample volume per day (~ 2 mL) means it was vital to optimize various parameters to maximize the amount of data that could be acquired for each sample preparation:

1. *The dead volume* required for sample to reach the optical window (tubing length / diameter, cell attachments, window area, etc) was minimized while still enabling bubble-free, uniform flow and for the flow setup to interface with the optical setup (*Fig. 1*) while accounting for a rastering (*i.e.*, moving) cell.

2. *A flow rate* of 0.9 mL h^{-1} was found to achieve an optimized balance between maximizing coverage by the laser of the sample when within the optical window and the acquisition of data that did not vary because of photodegradation between each scan in.

3. *The cell was rastered* during data acquisition using a Lissajous pattern to both limit photodegradation and further improve coverage by the laser of the flowing sample.

Because of limited CarH sample, these parameters were optimized in the first instance using a solution of free AdoCbl (*Fig. 2*). It is clear that high quality, reproducible data can be acquired at AdoCbl concentrations similar to those used in the CarH experiments.

In the context of this method, the fact that the CarH photoproduct precipitates is in fact a benefit. Despite our efforts to maximise sample coverage by the pump and probe beams, the nature of TRIR spectroscopy means that only a relatively small amount of sample is photo-converted during a single 'pass' through the optical cell (*Fig. 3A*). The precipitated photoproduct can therefore be removed from the collected sample by centrifugation and the supernatant recycled for subsequent data acquisition (*Fig. 1*). Although this population of photoconverted precipitate is enough to result in problematic scatter, it is not enough to reduce the sample concertation by a substantial amount after removal. Thus, the TRIR signal quality during at least one subsequent data acquisition cycle is not adversely impacted (*Fig 3B*). This means data from two 10-cycle passes can be averaged to improve signal to noise.

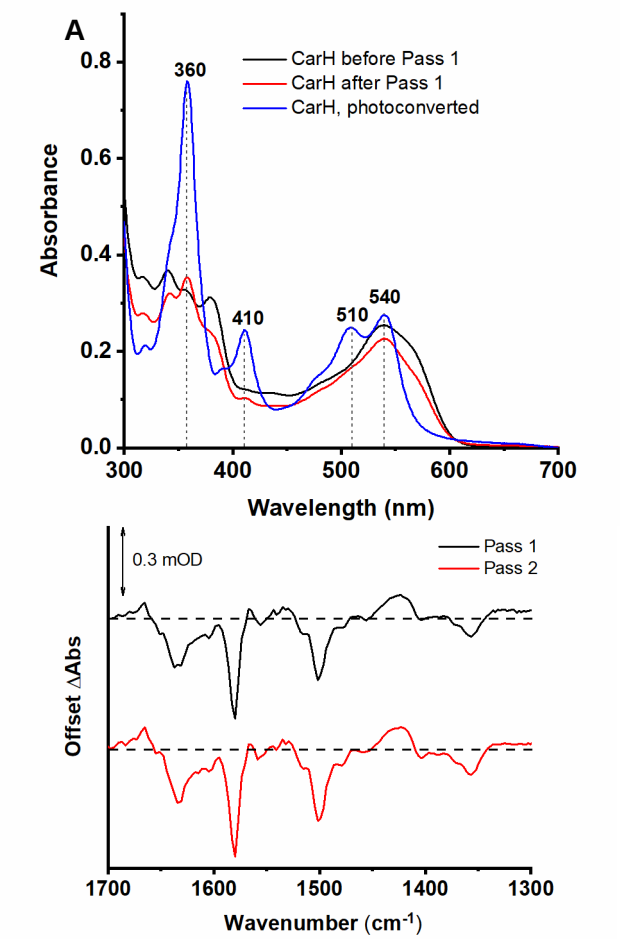

Figure 3A. *UV-vis spectra of CarH before and after one TRIR data acquisition 'pass', and fully photoconverted. Only a small proportion is converted after one 'pass'.* ***B.*** *TRIR difference spectra at 2 ps following photoexcitation of CarH at 525 nm in aqueous buffer. The data illustrate the reproducibility of sequential acquisition 'passes' following centrifugation as shown in Fig. 1. Plots are offset for clarity; dashed lines indicate zero spectral amplitude; and a scale bar is indicated.*

Conclusions

Photorecceptor proteins play a pivotal role in adaptation and survival across nature and have significant potential to form the basis of light-activated tools and therapies that offer unrivalled spatiotemporal control. For this potential to be realized, it is crucial that we gain a comprehensive, time-resolved understanding of their mechanism, structure and function. TRIR has an important role to play, but not all proteins are straightforwardly amenable to investigation using this method. Here, we demonstrate that the sample handling, flow and data acquisitions methods can be optimized for a 'tricky' photoreceptor, in this case the bacterial transcriptional regulator, CarH. By turning an ostensible barrier – *i.e.*, precipitation of the photoproduct – into an advantage, we have been able to recycle limited and costly sample in order to improve the quality of small signals. We hope that this system of approach can be adapted for other challenging samples and thus further enhance the utility of TRIR for biophysical study.

Acknowledgements

IC and ARJ thank Department for Business, Energy and Industrial Strategy for funding.

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