



Research paper

Photocycle populations with femtosecond excitation of crystalline photoactive yellow protein



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ARTICLE INFO

Article history:

Received 14 February 2016

Revised 4 April 2016

In final form 28 April 2016

Available online 30 April 2016

ABSTRACT

We investigate photocycle excitation of crystalline photoactive yellow protein using femtosecond laser pulses. This work establishes the feasibility and suitable optical excitation conditions to perform femtosecond time resolved X-ray crystallographic measurements using an X-ray free electron laser. Flash photolysis experiments demonstrated photocycle yields of the long-lived 'pB' signalling state of PYP of up to 10% with pulse durations of 130, 500 and 850 fs at 450 nm wavelength. The power density dependence of the transient pB concentration depends strongly on the pulse duration primarily because photobleaching is prominent at the GW/mm² level.

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1. Introduction

Femtosecond structural dynamics are traditionally measured using laser spectroscopy methods. It has recently become possible to directly detect ultrafast structural motion with near-atomic resolution and sub-picosecond time resolution [1,2]. Time resolved pump–probe X-ray crystallography measurements conducted at synchrotrons have previously been demonstrated for myoglobin-CO and Photoactive Yellow Protein (PYP) with a time resolution as low as 100 ps [3–9]. X-ray free electron lasers (XFELs) provide femtosecond hard X-ray pulses which can be used for ultrafast protein X-ray crystallography [1,2,10,11]. The serial femtosecond crystallography (SFX) method has emerged as the principal technique to exploit the short pulse duration for pump–probe time resolved crystallography measurements [1,2,12], rather than single-crystal experiments [13]. XFELs such as the Linac Coherent Light Source (LCLS) can provide pulses with a duration as low as 20–80 fs with X-ray energies of 4–10 keV for the fundamental and up to 25 keV for the third harmonic [14]. XFELs possess an inherent temporal jitter (given as ≈ 200 fs [15] at LCLS) which previously limited the time resolution that could be achieved by pump–probe schemes with synchronised optical lasers. Recent improvements in timing tools allow higher temporal resolution through accurate measurement of each shot's arrival time followed by time-sorting during processing [16]. This has opened up the possibility to study

dynamics of structural changes within protein crystals on previously inaccessible time scales < 1 ps.

In order to successfully detect small photoinduced differences in structure factor amplitudes, it is essential that a significant percentage of the probed crystal volume is photoexcited. In the case of excitation by intense femtosecond optical pulses, optical penetration is dominated by the non-linear cross sections, however populations also have a strong dependence on power density. A further complication is that multi-photon ionisation rapidly becomes dominant for most biological molecules, typically in the GW/cm² region [17–20], as also shown in this study. The most appropriate optical conditions for a time-resolved X-ray crystallography experiment must therefore be a trade-off between power density and peak power, which establishes hard limits on the experimentally available time resolution and on the maximum levels of detectable populations. Here, we investigate the yields and photobleaching processes in PYP samples in crystalline form, and extract the effective non-linear cross sections.

The maxima of the ground state absorption band of PYP (S_0) is at 449 nm in crystals. Upon single excitation ($S_0 \rightarrow S_1$) from this state PYP can enter into a fully reversible photocycle reaction (shown in Fig. 1(a)). The first stage of the photocycle is photoisomerisation of the trans-coumaric acid (pCA) chromophore, which has a time constant reported in the 0.4–1.5 ps range [21,22] and a primary quantum yield approximately 20% [23]. Many femtosecond spectroscopy studies have characterised the primary photochemical reactions of PYP [17,23–31]. Fig. 1(b) summarises a scheme of the spectroscopically distinguishable states, adapted from Jung et al.

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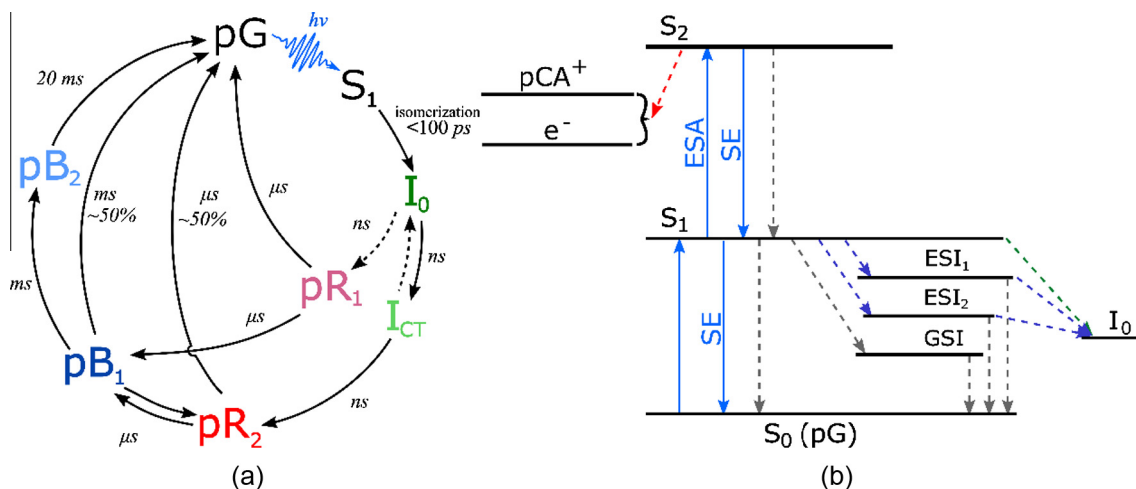


Fig. 1. (a) A kinetic scheme for the photocycle of wild-type PYP in crystalline form showing the more stable intermediates and the time scales between the formations of each. (b) A simplified ground state energy level diagram. Optically driven transitions and relaxations transitions are shown by solid and dashed arrows respectively. Photocycle entry is achieved by relaxation of $S_1 \rightarrow I_0$ either directly (green) or via an excited state intermediate (ESI) (purple). ESA results in double excitation (S_2) which can lead to the photoejection of an electron (e^-) and production of a p-coumaric radical (pCA^+).

[4]. The rates of excitation, stimulated emission (SE) and excited state absorption (ESA) will dictate the population of S_1 . These processes are influenced by the wavelength, duration, spectral phase and power density of the optical pump pulse. This work builds on previous investigations by Lincoln et al. [22] into the cross-sections and quantum yields of primary photochemical transitions of PYP in solution. They showed that optimised photoisomerisation yield with stretched femtosecond pulses has a significant dependence on the pump wavelength within the absorption band. For excitation at 400 nm the yield was limited by multiphoton absorption through excited state absorption (peak at 385 nm). This produces S_2 which can lead to photo-ionisation and the production of a pCA^+ radical and a hydrated electron [17]. The S_2 state has a relatively long recovery time (< 1 ms) and will therefore act as a loss of potential photocycle yield population during the time-scales of SFX measurements. Excitation at 490 nm greatly increases the effective SE cross section ($2.09 \times 10^{-16} \text{ cm}^2$) resulting in a high level of de-excitation ($S_1 \rightarrow S_0$) that also limits the rate of photo-isomerisation [22]. Pumping at 450 nm was found to be the best compromise to minimise both ESA and SE and therefore maximise the population excited to S_1 . Additional benefit was found from pulse stretching, however this comes at the expense of pump-probe time resolution. The conversion of S_1 to I_0 is still limited by the quantum yield of $\approx 20\%$ [22], which sets the absolute maximum yield that could be achieved with short pulses.

Those measurements and spectroscopically distinguishable states were obtained from solution experiments. Although the states are likely to be similar in crystals, the rates for their formation and decay, and hence their peak populations, may differ significantly in crystals and solution, due ultimately to the additional constraints imposed on each molecule in the crystal by the intermolecular contacts. These constraints favour the ground state in which the crystals were grown; and the crystallization medium often has unusual conditions of ionic strength and pH that may further alter the kinetics [32]. Hence, the best compromise in conditions for experiments on crystals must be determined afresh. In order to perform measurements on crystalline PYP the repetition rate must be reduced to 10 Hz [32], to allow full photocycle ground state relaxation (Fig. 1a) if the probe spot is not moved, which we also experimentally confirmed. 1 ms after excitation, the pump-probe difference spectrum shows a fully developed ground state (transient) bleach at 446 nm, and 449 nm in crystals [32] and has negligible contribution from the blue-shifted 'pB' state which absorbs maximally at 355 nm [22] and also 355 nm in crystals [32]. This has been confirmed to also be the case for crystals of PYP [32], therefore this contribution evaluates the yield of the millisecond intermediate 'pB' that can be achieved by single wavelength flash-photolysis measurements. It follows that a measurement of the ratio of transient absorption at 1 ms relative to ground state absorption is a direct and model-free measurement

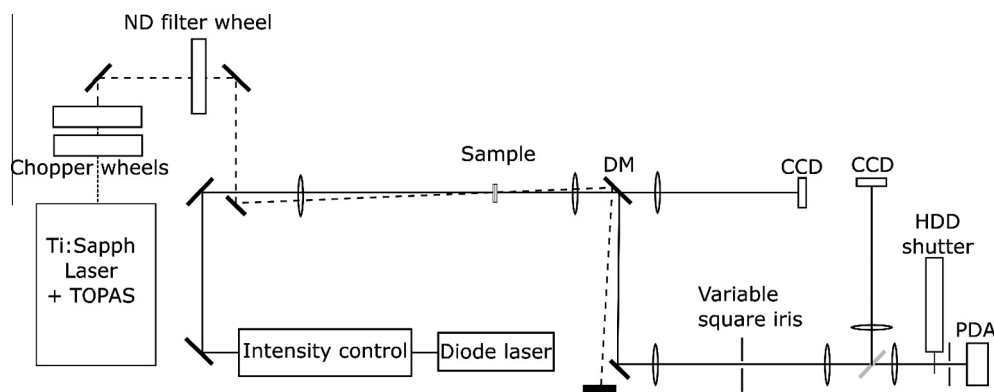


Fig. 2. Flash photolysis beam line set-up showing the femtosecond pump (dashed) and CW probe (solid). The probe is recorded on an amplified photo-diode (PDA).

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