Room temperature structures beyond 1.5 Å by serial femtosecond crystallography

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About 2.5 \times 10^6 snapshots on microcrystals of photoactive yellow protein (PYP) from a recent serial femtosecond crystallographic (SFX) experiment were reanalyzed to maximum resolution. The resolution is pushed to 1.46 Å, and a PYP structural model is refined at that resolution. The result is compared to other PYP models determined at atomic resolution around 1 Å and better at the synchrotron. By comparing subtleties such as individual isotropic temperature factors and hydrogen bond lengths, we were able to assess the quality of the SFX data at that resolution. We also show that the determination of anisotropic temperature factor ellipsoids starts to become feasible with the SFX data at resolutions better than 1.5 Å.

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INTRODUCTION

Photoactive Yellow Protein (PYP) is a blue light receptor in purple bacteria. It has been discovered in the 1980s (Meyer, 1985) and has since generated vast interest by a large group of researchers worldwide. After absorption of a blue photon, PYP enters a photocycle with numerous intermediates that accumulate and decay on multiple time-scales from picoseconds to seconds. The progression of the photocycle is controlled by a configurational change, a trans to cis isomerization, of the central chromophore para-cinnamic acid (PCA) that is covalently linked to Cys-69 of the protein (Fig. 1). The configurational change drives conformational changes of the protein with distinct intermediates. Before the photocycle comes to an end, the chromophore relaxes back to the trans configuration. Over the years, the photocycle has been extensively investigated with time-resolved crystallography on time-scales from 100 ps, the pulse duration of a synchrotron X-ray pulse, to seconds, when the reaction ends (Ihee et al., 2005; Jung et al., 2013; Schmidt et al., 2004; Schmidt et al., 2013; and Schotte et al., 2012). However, there is substantial interest to investigate the PYP reaction on ultrafast time-scales exploiting the femtosecond X-ray pulses at the Free Electron Lasers for hard X-rays (X-ray FELs). A proof-of-principle experiment has been recently (June 2014) performed on PYP at the Linac Coherent Light Source’s (LCLS) beamline CXI using time-resolved serial femtosecond crystallography (TR-SFX) at near atomic (1.6 Å) resolution with time-delays 10 ns and 1 μs after an optical pump laser pulse (Tenboer et al., 2014). The experiment shows that TR-SFX is feasible at an X-ray FEL. The door is now open to explore faster time-scales and potentially the femtosecond time-regime of the PYP photocycle. However, we anticipate that on the ultra-fast time-scale, structural changes are small and meaningful details can only be obtained if resolution is high enough. Here, we make use of the roughly 2.5 \times 10^6 snapshots that were collected during the

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June 2014 experiment in the dark to extend the resolution to 1.46 Å. We refined the PYP structural models against the SFX data at this resolution, and compared sensitive parameters such as temperature (B-) factors and hydrogen bond lengths to those derived from models that were obtained from in-house X-ray sources at near atomic resolution or synchrotron X-ray sources at atomic resolution.

METHODS

Methods are published earlier (Tenboer et al., 2014). In brief: PYP micro-crystals were grown from highly concentrated PYP. The crystals were about 5 µm long needles with 2 µm diameter (Fig. 2(a)). The suspension (5 × 10¹¹ crystals/ml) was jetted into vacuum by a gas dynamic virtual nozzle (Weierstall et al., 2012). FEL X-ray pulses of 40 fs duration containing about 10¹¹ photons/pulse were directed on the jet about 75 µm from the nozzle exit. The temperature of the jet was not controlled. However, since the jet velocity is about 10 m/s, the 75 µm path through the vacuum of the chamber until it is intercepted by the X-rays translates to about 7.5 µs. Assuming a cooling rate of 10⁶ K/s, the jet cools about 7.5 K. Hence, the temperature at the X-ray FEL beam intersection can be estimated to be about 15 °C, which is close to ambient (room) temperature. Diffraction patterns were collected by a Cornell SLAC Pixel Area Detector (CSPAD) located 75.1 mm from the jet. The center of the CSPAD was
electronically attenuated by about a factor of \( \sim 7 \) to allow for an increase in the dynamic range that allows the simultaneous collection of low and high resolution reflections (Fig. 2(b)). With this setup, about \( 2.5 \times 10^6 \) diffraction patterns (snapshots) were collected in the dark (Table I). Snapshots containing Bragg reflections were identified by CHEETAH (Barty et al., 2014). Indexing and integration was done by CrystFEL (White et al., 2012). The indexing ambiguity of the hexagonal PYP crystals was solved by methods (Brehm and Diederichs, 2014) implemented in CrystFEL.

Refinement was carried out with “REFMACS” (Murshudov et al., 2011) using the “log-likelihood residual.” B-factors were averaged for individual amino acid residues by “MOLEMAN” (Kleywegt et al., 2001) over the backbone atoms N, C\(_2\), C, and O. These B-factors \( \langle B \rangle_{\text{ind}} \) were plotted as a function of residue number. The trans/cis isomerization of the chromophore is controlled by three hydrogen bonds (Fig. 3(a)). Two extend from the phenolate oxygen (O4) of the chromophore head to O\text{d} of Glu-46 and O\text{g} of Tyr-42, and the third hydrogen bond is formed by the carbonyl-oxygen of the chromophore foot and the peptide bond nitrogen of Cys-69. The lengths of the O4-O\text{d} and O4-O\text{g} hydrogen bonds might depend also on the bond length of the chromophore ring C4’ to the hydroxyl oxygen O4 (Fig. 3(a)), which is set to the relatively large value 1.362 Å by a restraint in the “REFMACS” library file “mon_lib.cif.” If the X-ray data are weak and low resolution, this restraint enforces short hydrogen bonds. However, we tested this by setting the restraint to 1.238 Å, which is typically found in older “CNS” (Brunger et al., 1998) and “XPLOR” (Brunger et al., 1990) parameter files. This should bias the refinement towards longer hydrogen bonds. Refinement was carried out with either restraints (see below). The lengths of the hydrogen bonds were measured by the program “COOT” (Emsley et al., 2010) and compared to those found within existing structures in the literature.

RESULTS

The SFX data were integrated to a resolution of 1.37 Å. However, the \( R_{\text{split}} \) is unacceptable (84.3%) at this resolution, and it is difficult to use it to determine a resolution limit. The I/sigI (Table I) which is frequently used in crystallography to determine the resolution limit is differently determined in SFX due to the lack of profile fitting, the large shot-to-shot intensity variations as well as spectral fluctuations of the FEL X-ray beam (Karplus and Diederichs, 2012; White, 2014; and White et al., 2012). In order to estimate that limit, we used Fourier Shell Correlation (FSC), Eq. (1), which is output by REFMAC5 (Brown et al., 2015). The FSC is routinely employed in electron microscopy where it is the “gold-standard” to estimate the resolution limit (Scheres, 2012). The FSC was calculated to 1.37 Å in 25 resolution bins (Eq. (1)) and plotted as a function of \( 4 \sin^2 \theta/\lambda^2 = \frac{1}{d^2} \), where \( d \) is the resolution

\[
FSC = \frac{\sum_{h} F_{\text{obs}}^h F_{\text{calc}}^h}{\sqrt{\sum_{h} F_{\text{obs}}^h} \sqrt{\sum_{h} F_{\text{calc}}^h}^2}
\]

\( (1) \)

| TABLE I. Data collection statistics (last resolution shell: 1.49–1.46 Å). |
|-----------------|-----------------|
| Diffraction pattern | 2,463,000       |
| Hits             | 170,853 (7% of total) |
| Indexed          | 118,260 (69% of hits, 4.8% of total) |
| Resolution       | 1.46 Å          |
| # unique reflections | 16,787       |
| Competence [%]   | 100             |
| I/sigI           | 9.0 (0.3)       |
| \( R_{\text{split}} \) (%) | 6.89 (56.7)  |
To evaluate the resolution limit, we refined a PYP model with increasing resolution limits and stopped increasing the resolution when the FSC between the observed SFX amplitudes ($|F_{\text{obs}}|$) and those calculated from the model ($|F_{\text{calc}}|$) falls below 50%, which happened at 1.46 Å (Fig. 4). For the refinement, we started with the original PYP model (Borgstahl et al., 1995) with PDB (Berman et al., 2002)-entry 2PHY. We removed the double conformations in 2PHY and the water molecules. After rigid-body and restrained refinement, water molecules were added and double conformations were identified using Fo-Fc difference electron density maps. The refinement settles at 16.5% $R_{\text{cryst}}$ and 20.1% $R_{\text{free}}$ (Table II). The average B-factor is 13.5 Å$^2$. Anisotropic B-factor refinement decreases the R-factors further to 11.9% and 18.1%, respectively (Table II). Isotropic temperature factors averaged over the backbone atoms, ($B_{\text{inst}}$), are shown in Fig. 5(a) (black line). Hydrogen bond distances of the chromophore head to Glu-46 and Tyr-42 are 2.54 Å and 2.49 Å, respectively (Table II).

**DISCUSSION**

The number of unique reflections at 1.46 Å (16787, Table I) determined from the ~$2.5 \times 10^6$ SFX snapshots enables the refinement of an anisotropic B-factor model. The
resulting thermal ellipsoids are shown in Fig. 3(b). Notably, R_{cryst} and R_{free} factors decrease substantially. Obviously, the PYP structural model that includes anisotropic temperature factor ellipsoids is superior to the one with only isotropic B-factors. In Fig. 3(c), the thermal ellipsoids from a PYP structure refined to 0.82 Å with conventional macromolecular crystallographic (MX) data at cryogenic temperatures are shown (for the model see below, and Table II). The ellipsoids hardly differ regardless whether SFX or ultra-high resolution data are used. This is an indication that SFX provides adequate data for such a refinement. The gap between R_{cryst} and

![Figure 4](image)

**FIG. 4.** Fourier Shell Correlation and R_{split} as function of 1/d^2 (d is resolution). Open triangles and dashed-dotted line: FSC_work, solid squares and dashed line: FSC_free, solid line and spheres: R_{split}. Vertical long dashed line: 1.46 Å resolution.

| Table II. Refinement statistics, B-factors, and hydrogen bond lengths. H1: PCA-phenolate oxygen to Glu-42 Oδ. H2: PCA-phenolate O to Tyr-42 Oγ. H3: PCA-carbonyl O1 to Cys-69 N. All studies were performed on hexagonal P63 wt-PYP crystals with unit cell parameters (depending on temperature) around a = b = 66 Å, c = 40 Å. |
|-------------------------------------------------|
| pdb | PYP_{SFX} this study | PYP_{B293} | PYP_{A293} | PYP_{A110} | PYP_{G149} |
|---|-----------------|---------|---------|---------|---------|
| X-ray source | LCLS | rotating anode | APS | APS | SSRL |
| Method | SFX | rotation | rotation | rotation | rotation |
| Crystal size | 2 x 2 x 5 μm^3 | 250 x 250 | 180 x 180 | 180 x 180 | 200 x 200 |
| | x 1000 μm^3 | | x 500 μm^3 | | x 800 μm^3 |
| T (K) | 288^a | 293 | 293 | 110 | 149 |
| Resolution (Å) | 1.46 | 1.4 | 1.10 | 1.00 | 0.82 |
| Refine | REFMAC5 | XPLOR | SHELX97 (Sheldrick, 2008) | SHELX97 | SHELX97 |
| (B) (Å^2) | 13.5 | 11.7 | 19.4 | 12.1 | 11.3 |
| B_{iso} | yes | no | yes | yes | yes |
| R_{cryst}/R_{free} (%) | 16.5/20.1^b | 18.6/22.6 | 13.3/16.1 | 13.4/16.1 | 12.3/14.4 |
| Hydrogen bonds (Å) | 11.9/18.1^c |
| H1: Glu-46 | 2.54/2.55^d | 2.69 | 2.58 | 2.59 | 2.58 |
| H2: Tyr-42 | 2.49/2.49^d | 2.71 | 2.51 | 2.50 | 2.48 |
| H3: Cys-69 | 2.78 | 2.69 | 2.82 | 2.75 | 2.72 |

^aEstimated.

^bIsotropic B-factor refinement.

^cAnisotropic B-factor refinement.

^dLong C4’–O4 bond restraints/short C4’–O4 restraints.
R_free indicates a certain degree of overfitting, which is, however, acceptable in the light of the improved refinement statistics.

In Table II, five different PYP models are compared: PYP_{SFX} (this study), PYP_{B293}, the original model determined by the Getzoff group (Borgstahl et al., 1995), PYP_{A293} and PYP_{A110}, which were both determined at atomic resolution by Anderson and colleagues (Anderson et al., 2004) at room temperature (293 K) and cryogenic temperatures (110 K), respectively, and PYP_{G149} which has been determined to ultra-high resolution (0.82 Å) by Genick and coworkers (Getzoff et al., 2003) at 149 K. Hence, two room temperature structures and two structures determined at cryogenic temperatures are available and can be compared to PYP_{SFX}. PYP_{A293} has the highest overall B-factor, \( \langle B \rangle \). To reach 1.1 Å resolution, exposure times to the synchrotron X-rays had to be long. Accordingly, the absorbed X-ray dose is quite high, especially with the relatively small crystals (Table II). Since at room temperature PYP does not show specific local damage (Schmidt et al., 2012), the high B-factor reflects global damage. In Fig. 5(a), the temperature factor averaged over individual residue back bone atoms \( \langle B \rangle_{\text{ind}} \) is plotted as a function of the residue number. The \( \langle B \rangle_{\text{ind}} \) for the PYP_{A293} (red line) are larger than those for PYP_{SFX} (black line) and PYP_{B293} (blue line). The global damage is revealed by the offset of the red curve with respect to the others. However, for PYP, the global damage does not affect the functionality (the kinetic properties) of the PYP ensemble in the crystal (Schmidt et al., 2012). Despite the potentially higher B-factor, the PYP_{A293} structure is functionally relevant. The smallest B-factor at room temperature is found in the original model PYP_{B293}, which was determined with relatively large crystals and correspondingly low absorbed dose on a home source (Borgstahl et al., 1995). Its \( \langle B \rangle \) is as small as those \( \langle B_s \rangle \) determined at cryogenic temperatures in PYP_{A110} and PYP_{G149}.

When crystals are frozen, the structural distribution and integrity is maintained even at higher doses (Garman and Weik, 2011; Owen et al., 2006), and resolution as high as 0.82 Å can be reached without noticeable radiation damage. The PYP_{SFX} structure on the other hand shows also low B-values, which are close to the cryogenic structures and the in-house model. This is yet an additional proof that SFX is, due to the “diffraction-before-destruction” principle (Chapman et al., 2011), (almost) free of radiation damage even at room temperature, although doses that are deposited in the tiny crystals may be orders of magnitude higher than the safe dose (Lomb et al., 2011). It can be speculated that the small difference between the \( B \) of the original model PYP_{B293} (Fig. 5(a), blue line) and that of PYP_{SFX} (Fig. 5(a), black line) reflects...
some crystal disintegration during the end of the 40 fs X-ray FEL pulse that may be precluded by using even shorter X-ray FEL pulses. The (B)_i^ind of the PYPSFX structure are compared to those of the cryogenic structures in Fig. 5(b). The (B)_i^ind are almost identical although the structures were determined at different resolutions, at different temperature settings, and from very different crystal sizes. There are notable differences, though, between the cryogenic structures and PYPSFX around residues 16–22, 80–88, and 112–117. All three moieties are loops with little crystal contacts (see Figs. 1 and 5(b), red bars). Their motions can be frozen out by lowering the temperature which increases the Debye Waller factor and the scattering power into the Bragg reflections at low temperatures. The mentioned loop from residue 112–117 might play an important role to mitigate the signal from the chromophore pocket to the periphery of the protein (Ihee et al., 2005; Tenboer et al., 2014).

Hydrogen bonds are important for the functionality of the PYP chromophore (Anderson et al., 2004). They fix the chromophore head between Glu-46 and Tyr-42 (Fig. 3, and Table II). In the original model PYPB293, no unusual hydrogen bond lengths were reported (Table II). The high resolution room and low temperature structures unexpectedly showed that the hydrogen bonds are much shorter (Table II), which supports the idea of a chromophore configuration that is ready to move. Short hydrogen bonds are energetically unfavorable. The chromophore head must be pressed into the hydrogen network due to interactions with amino-acids of the chromophore pocket. Without pocket relaxation the head is tightly kept in position. When a blue photon is absorbed, a trans to cis isomerization occurs. Since at very early times (100 ps to 1 ns) the pocket relaxations are very limited (Jung et al., 2013; Schotte et al., 2012), the chromophore head cannot move. Instead, the tail rotates (Ihee et al., 2005; Jung et al., 2013; and Schotte et al., 2012). However, when the pocket relaxes at longer times on the μs to ms time-scale (Ihee et al., 2005; Schmidt et al., 2013), the chromophore is ready to snap out of its position. In addition, the putative signaling pB state is very long lived (Schmidt et al., 2013), because the formation of the dark state requires the formation of this tightly knit chromophore pocket. The structural refinement against the SFX data corroborates this view. The hydrogen bonds are short (Table II), irrespective of whether long restraints for the C4'-O4 bond (1.362 Å) or short restraints (1.238 Å) are employed. The hydrogen bonds are much alike to those observed in the highest resolution structures. Moreover, even when the C4'-O4 bond is biased by the short 1.238 Å restraint, it elongates by 0.11 Å to 1.347 Å. This result is also obtained from the highest resolution structures emphasizing again the excellent quality of the SFX data.

Our results show that SFX determines structures in their functional form which are comparable to those determined at both room temperature and cryogenic temperatures at highest resolutions possible. Unlike summarized recently in a review (Schlichting, 2015), the quality of our SFX data is superb. Synchrotron like data quality and resolution are reached at room temperature without suffering from radiation damage. The attenuation mask (Fig. 2(b)) allowed the accurate determination of low resolution intensities without impairing the quality of the high resolution data. Furthermore, the CSPAD tile geometry (Fig. 2(b)) can be exquisitely well determined by refining individual tile positions with help of the X-ray data (Tenboer et al., 2014). All of this resulted in highly accurate amplitudes even in the presence of an indexing ambiguity and without introducing partiality models (Kabsch, 2014; Sauter et al., 2013). For the collection of the ~2.5 × 10⁶ snapshots, about 6 h of FEL beamtime is necessary. With an average jet flow of 22 μl/min, about 7.6 ml of crystal slurry is dissipated which contains roughly 500 mg of microcrystalline protein. It is desirable, therefore, to reduce the number of required X-ray snapshots with computational methods (Sauter, 2015; Uervirojnangkoorn et al., 2015; and White, 2014) to obtain comparable data quality within much shorter beamtimes, collecting a much smaller number of snapshots from a much lower amount of protein.

**SUMMARY**

We push the resolution of macromolecular serial femtosecond crystallography to 1.46 Å. Anisotropic B-factor refinement starts to become possible with SFX data at that resolution. The
structures show little to no evidence of radiation damage even at ambient temperatures and are comparable to ultrahigh (atomic) resolution structures.

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