

structure-factor amplitudes reported for  $I_T$  (PDB ID: 4I38), for which  $R_{\text{crist}} = 0.2266$  when the three dihedral angles between the pCA phenolate and carbonyl were fixed at the values found in the X-ray structure of Jung *et al.*, versus  $R_{\text{crist}} = 0.2267$  when fixed at the corresponding values in their DFT structure. The electron density maps in Fig. 1a,b were extrapolated from low-occupancy (<10%) difference maps, and lack the spatial resolution required to refine the coordinates of individual pCA chromophore atoms without chemical restraints. During their X-ray structure refinement, Jung *et al.* gradually released the dihedral angle restraint across the  $C_3=C_2$  bond and recovered a structure whose central dihedral angle deviated significantly from that found in their DFT structure. Using the DFT methods reported by Schotte and colleagues<sup>2</sup>, we found the  $I_T$  X-ray structure of Jung *et al.* to be unstable: it converged to a conformation similar to their  $I_T$  DFT structure and the  $pR_0$  structures of Schotte *et al.* (Fig. 1D).

According to the kinetic model of Jung *et al.*,  $I_T$  bifurcates into  $I_{CT}$  (PDB ID: 3VE4/4I39) and  $pR_1$ , with  $I_{CT}$  supplanting the planar *cis* intermediate reported in prior time-resolved X-ray (PDB ID: 4BBT, 1TS8)<sup>2,5</sup> and cryo-crystallography studies (PDB ID: 1OT9, 1UWP)<sup>6,7</sup>. This model implies that the well-characterized planar *cis* intermediate, denoted  $I_{CP}$  by Ihee and colleagues<sup>5</sup>, does not exist. The opposite seems more plausible: we performed DFT calculations on  $I_{CT}$  and found that its structure was unstable, and converged to a

conformation similar to the DFT structures reported for  $I_T$  and  $pR_0$  (Fig. 1d). Jung *et al.* invoked  $I_{CT}$  to explain persistence of a twisted intermediate following the decay of  $I_T$ , whereas Schotte *et al.* accounted for this persistence with a reversible transition between  $pR_0$  and  $pR_1$  (in the notation of Schotte *et al.*,  $pR_1$  is similar to the intermediate  $I_{CP}$ ), a view that is supported by their similar DFT energies<sup>2</sup>. Had Jung *et al.* allowed for this reversibility, they could have accounted for their time-resolved electron density maps with  $I_{CP}$ , whose structure is supported by both DFT and prior crystallography studies<sup>2,5</sup>.

How might differences in the buffer conditions influence the results? In ~2.8 M ammonium sulfate, two long-lived  $pR$  intermediates were required to account for the time-resolved diffraction data<sup>1,5</sup>. In 1.1 M NaCl and 2.5 M ammonium sulfate, which is arguably more physiologically relevant for halophilic bacteria, only one long-lived  $pR$  intermediate was required<sup>2</sup>. Whereas 1.1 M NaCl seems to simplify the PYP photocycle, it is difficult to rationalize how its absence could stabilize  $I_T$  in a high-energy twisted state, or morph planar  $I_{CP}$  into an unstable, twisted  $I_{CT}$  conformation.

In conclusion, the contradiction between Jung and colleagues' X-ray and DFT structures for  $I_T$  arises from an empirical choice to loosen rather than tighten their dihedral angle restraint across the  $C_3=C_2$  bond. This choice seems not to be compelled by their diffraction data, and results in an X-ray structure that is more consistent with the electronic excited state.

If we make the more plausible assumption that  $I_T$  is a ground-state intermediate, the unveiling of "... a long-hypothesized highly twisted intermediate along the *trans*-to-*cis* isomerization pathway" has not yet occurred, but may prove possible with a free-electron X-ray laser such as the Linac Coherent Light Source, where sub-ps time resolution can be achieved. □

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## Reply to 'Contradictions in X-ray structures of intermediates in the photocycle of photoactive yellow protein'

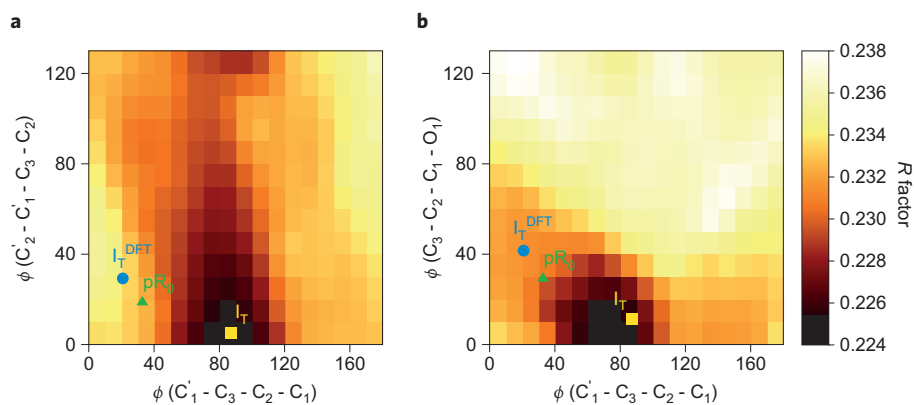
**Jung *et al.* reply** — We recently reported picosecond time-resolved crystallographic investigations on photoactive yellow protein<sup>1</sup>. Now Schotte and colleagues challenge<sup>2</sup> the structural interpretation of our results based on their work<sup>2,3</sup>. In particular, they disagree with structural details of our earliest intermediate  $I_T$  and the next intermediate  $I_{CT}$  based on their density functional theory (DFT) calculations. We stand by our results because, for both intermediates, the time-resolved X-ray data and the experimental electron densities favour the structures that we reported over the structures derived from DFT.

Our study<sup>1</sup> and that by Schotte *et al.*<sup>3</sup> used the same experimental technique but differ in that (1) in our work we also studied the E46Q mutant as well as the wild-type

(WT) protein, (2) the crystals were grown under quite different conditions, and (3) the X-ray data quality and crystallographic completeness differ.

Figure 1 shows the dependence of the  $R$  factor on three dihedral angles. Whereas  $I_T$  is located at the minimum  $R$  factor, both the DFT structure ( $I_T^{\text{DFT}}$ ) and the earliest structure of Schotte *et al.*<sup>3</sup> ( $pR_0$ ), which supposedly corresponds to  $I_T$ , are far from the minimum. Notably, in the 'Structure Refinement' section of the Supporting Information of their paper, they report that  $pR_0$  also tends to adopt a  $C_1'-C_3-C_2-C_1$  dihedral angle close to 90°, as found in  $I_T$  when the structure was not restrained to resemble  $I_T^{\text{DFT}}$ . Thus, the refined dihedral angle varies depending on whether the structure is restrained to mimic  $I_T^{\text{DFT}}$  (their approach) or is allowed to follow

the experimental electron density (our approach). Our approach was to compare the qualitative features of  $I_T^{\text{DFT}}$  and  $I_T$  instead of using  $I_T^{\text{DFT}}$  as the structural restraints. Although  $I_T$  obtained without such restraints has a different dihedral angle from that of  $I_T^{\text{DFT}}$ , we were content with the fact that  $I_T^{\text{DFT}}$  also supports a non-planar structure consistent with  $I_T$ . We believed that forcing the structural refinement of the experimental density to meet the restraints from the DFT structure removes the possibility that the experimental data could provide any new information other than the boundary given by DFT. Strictly speaking, no new information was obtained in their study, and such an approach would always yield only those structures compatible with DFT even when new experimental data



**Figure 1** | The dependence of the  $R$  factor on the three dihedral angles ( $\phi$ ). **a**,  $C_2-C_1-C_3-C_2$  versus  $C_1-C_3-C_2-C_1$  and **b**,  $C_3-C_2-C_1-O_1$  versus  $C_1-C_3-C_2-C_1$ . Whereas  $I_T$  is located at the minimum  $R$  factor,  $I_T^{\text{DFT}}$  and  $pR_0$  are far from the minimum. The same situation is found also for  $I_{CT}$  versus  $I_{CP}$  (data not shown).

with better temporal and spatial resolution become available from experiments at X-ray free-electron lasers. The possibility of interactions that stabilize such a highly strained dihedral angle but are not fully accounted for by DFT cannot be ruled out. In addition, because DFT is a single-reference-based method, DFT may not well describe such a highly distorted structure of the chromophore whose multiconfigurational character might be strong. It should also be noted that  $I_T$  is observed in both E46Q and WT with a dihedral angle  $C_1-C_3-C_2-C_1$  that is smaller in E46Q by  $\sim 15^\circ$ .

Regarding the choice between  $I_{CT}$  and  $I_{CP}$ : our maps in the present study are consistent with those Ihee *et al.* reported earlier<sup>4</sup>. In that earlier work, due to the limited time resolution, Ihee *et al.* used only a single structure,  $I_{CP}$ , to fit the maps at the time delays on the nanosecond timescale. This fit was only partly satisfactory and left some residual density. Further, the single  $I_{CP}$  structure does not have the minimum

$R$  factor. Our work in ref. 1 explains these observations: the maps in both the present and the earlier studies are structurally heterogeneous and contain  $I_{CT}$  and  $pR_1$ . Schotte *et al.*<sup>3</sup> explain this residual, non-planar density differently: they assume an equilibrium between the first and second intermediates, but this kinetic scenario gives a worse fit to our experimental densities for both WT and E46Q. Moreover, such an equilibrium is highly unlikely because, at early times, the chromophore is highly strained and the reaction is likely to proceed strongly downhill.

Although Schotte *et al.*<sup>3</sup> direct their major attention to the detailed structural features of the early intermediates, a more serious discrepancy between us is found even on the well-established microsecond time range. They identify<sup>3</sup> only one structural species ( $pR_2$ ) whereas our study<sup>1</sup> and others<sup>4,5</sup> reported that two species ( $pR_1$  and  $pR_2$ ) co-exist. It is not clear yet whether this discrepancy arises from the experimental conditions,

or from data analysis and interpretation. Our conditions of lower salt (50 mM NaCl) and neutral pH (pH 7.0) have been extensively used for earlier time-resolved X-ray crystallographic investigations of PYP. In contrast, Schotte *et al.* used crystals grown in high salt (1.1 M NaCl) and  $D_2O$  (pD 9.0), although their ammonium sulfate concentration ( $\sim 2.5$  M) was close to ours ( $\sim 2.6$  M). Because salt and pH may well affect the structure and dynamics<sup>6-8</sup>, the exact origin of these discrepancies remains to be studied. □

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