structure-factor amplitudes reported for I_T (PDB ID: 4I38), for which $R_{cryst} = 0.2266$ 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 $R_{cryst} = 0.2267$ 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[2], 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)[2,5] and cryo-crystallography studies (PDB ID: 1OT9, 1UWP)[6,7]. This model implies that the well-characterized planar cis intermediate, denoted I_{CP} by Ihee and colleagues[5], 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[2]. 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[2,5].

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[1,5]. 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[2]. 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[1]. Now Schotte and colleagues challenge[2] the structural interpretation of our results based on their work[2,3]. 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[1] and that by Schotte et al.[3] 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^{DFT}) and the earliest structure of Schotte et al.[3] (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^{DFT}. Thus, the refined dihedral angle varies depending on whether the structure is restrained to mimic I_T^{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^{DFT} and I_T instead of using I_T^{DFT} as the structural restraints. Although I_T obtained without such restraints has a different dihedral angle from that of I_T^{DFT}, we were content with the fact that I_T^{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
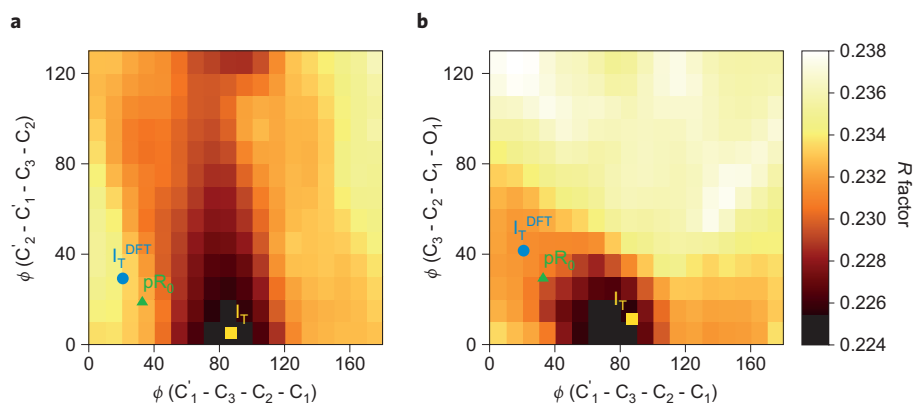
a

b

Figure 1 | The dependence of the *R* factor on the three dihedral angles (ϕ). **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^{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 ~15°.

Regarding the choice between I_{CT} and I_{CP}: our maps in the present study are consistent with those Ihee *et al.* reported earlier[4]. 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.*[3] 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.*[3] 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[3] only one structural species (pR_2) whereas our study[1] and others[4,5] 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 (~2.5 M) was close to ours (~2.6 M). Because salt and pH may well affect the structure and dynamics[6–8], the exact origin of these discrepancies remains to be studied. ❑

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