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Electronic Supplementary Information

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Steady-state fluorescence spectrum of M15

The fluorescence characteristics of M15 were investigated through dual-wavelength excitation at pH 8. Upon excitation at 400 nm, two emission peaks were observed: one at 450 nm corresponding to the emission from the SB excited state, and another at 650 nm attributed to the PSB state emission formed through ESPT. Selective excitation of the PSB state at 540 nm yielded a single emission peak at 650 nm, further confirming that this peak indeed originates from the PSB state emission.



Fig. S1 Steady-state fluorescence spectra of M15 under different excitation wavelengths. Both spectra were normalized to their respective maximum intensities.

M15 Dynamics with 540 nm Excitation

Figure S2 shows the transient absorption spectra of M15 under 540 nm excitation. The 2D pseudocolor map reveals two primary spectral features: an excited-state absorption (ESA) band centered at 500 nm and a stimulated emission (SE) band extending from 600 nm to 750 nm. The ESA signal appears relatively weak due to the overlapping intense SE signal in the same spectral region. According to the steady-state spectra, excitation at 540 nm predominantly probes the PSB state dynamics. This process exhibits similar characteristics to the dynamics observed for M3 under 400 nm excitation. However, it differs significantly from M15's own dynamics under 400 nm excitation, indicating that the mutation has effectively suppressed the proton transfer process.



Fig. S2 Femtosecond transient absorption spectra of M15 mutant at pH 8.0. Two-dimensional pseudo-color plot (left) and selected kinetic traces at different delay times (right) showing ESA (around 500 nm) and SE (>600 nm) features. The time-resolved spectra were recorded at various delay times (0.2, 5, 50, 300, and 800 ps) after 540 nm excitation. The color scale represents the change in optical density (Δ mOD) from -3 to 1.

Method Verification through Comparison with M15 Crystal Structure

To validate the reliability of our computational methodology, we first employed the M15 crystal structure as a test case and optimized its structure using the same modeling protocol. The optimized structure showed excellent agreement with the experi-

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Fig. S3 Structural validation and hydrogen bond analysis of M15. (A, C) Superposition of optimized structures (cyan) with crystal structure (wheat) for chains A and C. (B, D) Hydrogen bond networks around His40 in HIP (chain A) and HID (chain C) states, showing interactions with neighboring residues Q108K, Q128, and N13. Key hydrogen bond distances are indicated in Å.

mental crystal structure, exhibiting a backbone RMSD of approximately 1.2 Å, which demonstrates the accuracy of our computational approach in modeling overall conformations. Considering that histidine 40 (His40) has a pKa 7.6, its protonation state may vary under neutral conditions, potentially affecting the stability of the simulated structure and local interactions. To systematically evaluate this factor, we performed simulations with His40 in three common protonation states (HIP, HID, and HIE) using the same protocol. The results revealed that when His40 was set to HIP and HID states, the simulated structures showed the highest concordance with chains A and C of the crystal structure, respectively, with backbone RMSD values of approximately 1.2 Å and 0.92 Å. Both conformations successfully reproduced the internal hydrogen bond network observed in the experimental structure, indicating the robustness and reliability of our computational approach in handling microenvironment-sensitive residues and their hydrogen bond networks.

It should be noted that some discrepancies between the simulated and experimental structures were observed in the chromophore region, primarily due to the limited accuracy of the current small molecule force field in treating such conjugated systems. We employed the SMIRNOFF force field, a recently developed general-purpose force field based on direct chemical perception, which demonstrates good transferability.