Supporting Information

Protein-Bath Coupling of Internal Reaction Coordinate at Intermediate Timescales

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Methods

Sample preparation

eGFP (Biovision Inc., United States) was labeled using a biotin labeling kit (Roche, Switzerland) and purified. A solution of biotin-labeled eGFP (~0.5 mM) was incubated on a strepdavidin coated coverslip (Microsurface Inc, United States) for 60 minutes. Following incubation, the coverslip was rinsed with DI water to remove unbound eGFP. The coverslip was then mounted to a microscope slide and solvated with a buffer solution (PBS, pH 7.45, Thermofisher, United States). For viscosity dependent studies, mixtures of PBS buffer mixed with glycerol (Sigma Aldrich, United States) at volume ratios ranging from 0% to 30% by volume.

Single-molecule spectroscopy

Single-molecule imaging was carried out on a commercial Leica SP8 system (Leica, Germany). Photon-counting was performed by a SPAD detector equipped with TCSPC unit (PicoQuant, Germany). From the single-molecule photon stream containing roughly 5×10^6 photons, a series of 2D-FLCS spectra were computed using a 2D-inverse Laplace transformation (2D-ILT). The entire data analysis process is outlined in detail elsewhere.[1]

Fitting Correlation functions

The correlation functions are well fit to a single-exponential growth function. The average decay time, τ_{rxn}, is taken as the exchange time. The correlation functions are normalized according to Eq. 2 of the main manuscript.

$$C(\Delta t) = 1 - \exp\left(-\Delta t \middle/ \tau_{rxn}\right) \qquad \text{Eq. S1}$$
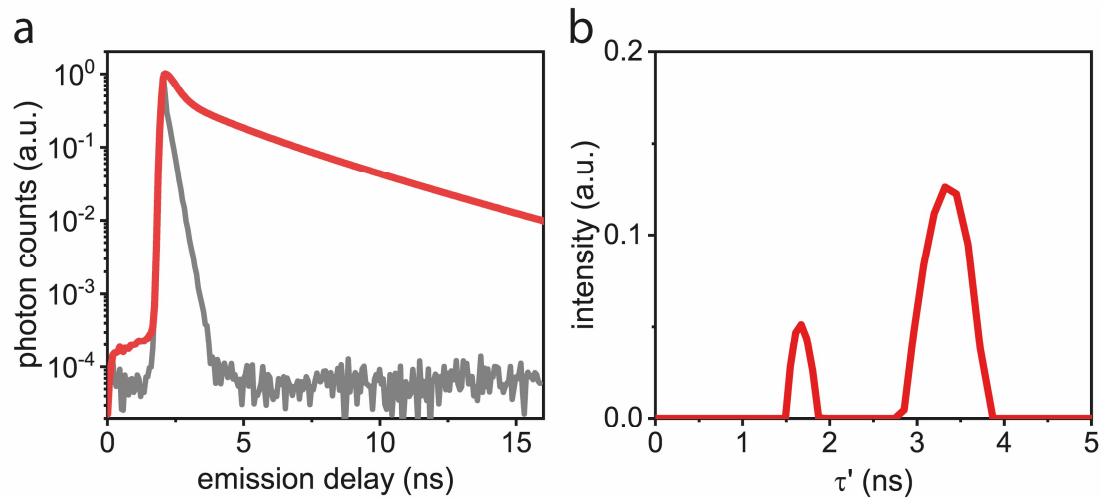
Figure S1. eGFP single-molecule fluorescence lifetime data. (a) Fluorescence emission delay histogram for eGFP (red data), shown with the instrument response function (grey data). (b) Corresponding 1D-ILT of the relaxation data, showing two peaks at $\tau_1 \sim 1.8$ and $\tau_2 \sim 3.2$ ns, corresponding to the two conformations of the chromophore pocket.
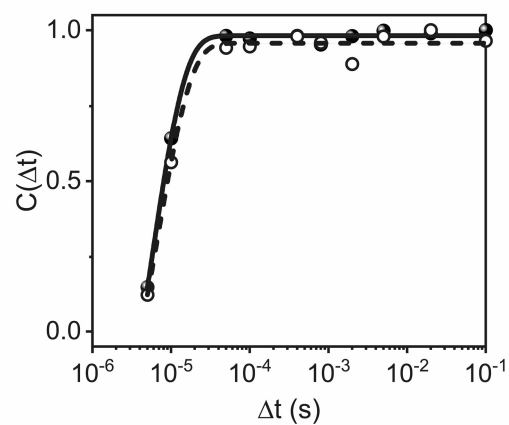
Figure S2. Forward and reverse exchange dynamics of eGFP in buffer. The forward and reverse reaction kinetics are extracted from opposite regions of the 2D spectrum. Under conditions of microscopic reversibility, the signal amplitudes are equivalent and the 2D spectrum is symmetric along the diagonal. As expected for equilibrium dynamics, the forward and reverse reaction kinetics are equivalent for the reaction occurring in the eGFP chromophore pocket. Fitting according to Eq. S1 are shown in solid and dashed lines.
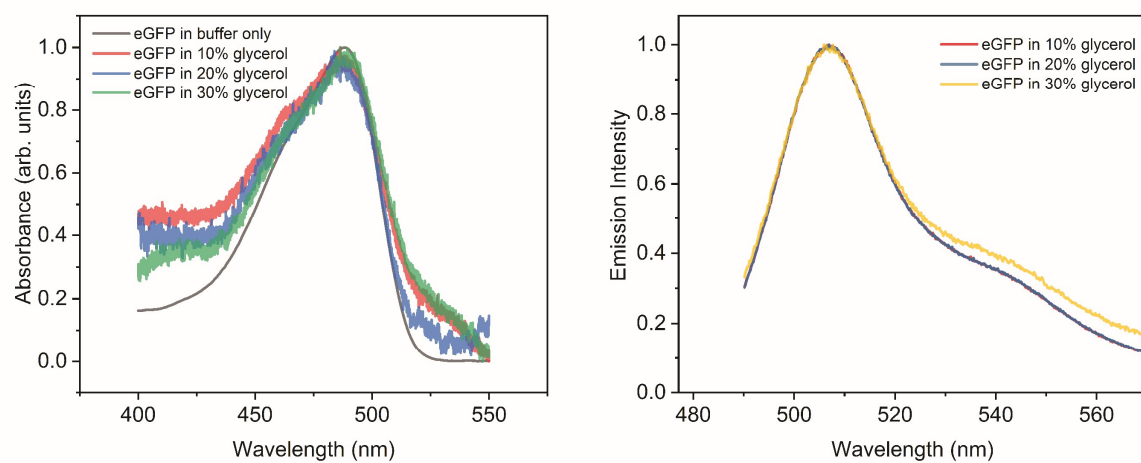
Figure S3. **Absorption and emission spectra of bulk eGFP under varying concentrations of glycerol.** Absorption and emission spectra show that the protein is folded at high glycerol concentrations.
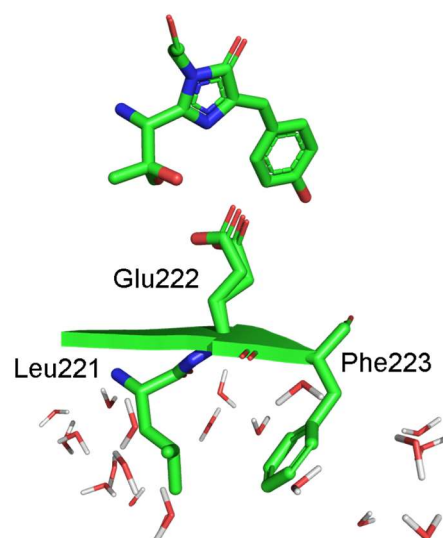
Figure S4. Interface between the interior and exterior residues coupled to the chromophore pocket. The Glu222 residue directly coupled to the chromophore pocket of eGFP, immediately flanked by two solvent-exposed residues (Leu221 and Phe223). Both neighboring residues are capable of dihedral rotation, and are thus likely to be strongly coupled to the bath. One possible mechanism for the observed viscosity dependence of the internal reaction of the chromophore pocket is an indirect coupled to the bath mediated by immediately adjacent residues along the protein backbone.

References

1. Talele, S.; King, J. T., Fast and Robust 2D Inverse Laplace Transformation of Single-Molecule Fluorescence Lifetime Data. *bioRxiv* **2021**.