Structural landscape of the isolated ligand binding domain of single AMPA receptors

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I. Supplementary Methods

Single Molecule FRET Sample Preparation and Data Analysis

Single GluR2-ABD immobilization was achieved via biotin-streptavidin-antiHIS-HIS chemistry sparsely dispersed atop a PEGylated glass coverslip surface. This both maximizes conformational freedom for the two lobes and minimizes undesirable, nonspecific protein/dye chemistry. Maleimide-conjugated Alexa555 and Alexa647 were attached to the GluR2-ABD molecule through two Cysteine residues that replace T394 and S652. The chosen labeling geometry has been determined to be free of unwanted dye-peptide interactions by measurement of ion conductance, comparison with alternate measurement/labeling schemes, and by dye emission lifetimes1. The resulting immobilized, dye-labeled single GluR2-ABD samples were topped with a micro-reaction chamber through which ionic and glutamate concentrations, as well as solution pH, were controlled via syringe pumps, as illustrated in Figure S1.

![Diagram of the scanning confocal microscope used in these experiments](image)

Figure S1. Dye-labeling and immobilization of GluR2-ABD
The GluR2-ABD was modified by inserting 8-Histidines(His8) at the N-terminus and by mutating two residues at position T394 and S652 to cysteine. Maleimide derivatives of Alexa 555 and 647 served as donor and acceptor probes. The single proteins were immobilized on glass coverslips using biotin-streptavidin-anti-HIS-HIS chemistry atop a PEGylated brush that reduces nonspecific protein-surface interactions.

A diagram of the scanning confocal microscope used in these experiments is shown in Figure S2. It is described more completely in the Methods section of the manuscript.

The first analytical challenge lies in that there are two inherent Cysteine residues (C425 and C436) that may be inadvertently dye-labeled, as illustrated in Figure S3. Although these have been shown to be largely inaccessible to fluorophore labeling using terbium chelate and fluorescein1, occasional multi-dye labeling was observed, characterized by multi-step
photobleaching, and eliminated from the data set. Table S1 includes the percentages of multi-dye labeled occurrences for each set of experiments.

Figure S2. Scanning confocal smFRET instrument.

The number of attached dyes can be deduced from the emission trajectory of a multilabeled molecule. The emission trajectory of a molecule with one donor and one acceptor shows a single-step photobleaching event for each of the donor and acceptor emissions (Figure S4A). If there are two donors and one acceptor in a molecule, its emission trajectory shows two-step photobleaching events for the donor emission (Figure S4A). Moreover, in the FRET region, one donor dye is transferring its energy to the acceptor while the other one is emitting photons, which means that it is farther than 10 nm from the acceptor dye. This results in a lower FRET efficiency. Generally, the presence of multiple dyes affects the calculated FRET efficiency values. Figure S4B is an emission trajectory where the acceptor emission undergoes two-step photobleaching events. Initially, the two acceptor dyes are emitting photons until one of them is photobleached. The other acceptor dye continues to fluoresce until both the donor dye and the remaining acceptor dye are photobleached. From Figure S4C, we can see that there are two donor and two acceptor dyes in the molecule where this emission trajectory was collected. There is a transfer of energy at first from the two donor dyes to the two acceptor dyes. When one acceptor dye is photobleached, the number of donor photons increased because one donor is not near enough to transfer its energy to the remaining acceptor dye while the other donor dye is still transferring its energy to the remaining acceptor dye. Figure S4D shows an emission trajectory of a donor only-labeled molecule.

Figure S3. GluR2-ABD molecules with multiple labels. (A) Molecule with two donor dyes and one acceptor dye. (B) Molecule with one donor dye and two acceptor dyes. (C) Molecule with two donor dyes and two acceptor dyes. (D) Molecule with two donor dyes.

Table S1. Percentages of labeled molecules

| Molecule | 1D:1A | 2D:1A | 1D:2A | 2D:2A |
|----------|-------|-------|-------|-------|
| Apo GluR2-ABD | 74% | 21% | 3% | 2% |
| GluR2-ABD + Glu | 78% | 17% | 4% | 1% |
| GluR2-ABD T686S + Glu | 86% | 14% | 0% | 0% |
The data for each protein system presented in the manuscript were acquired from 2-3 separate measurements. The data from the separate experiments were analyzed for reproducibility and other experimental factors such as initial/final smFRET quantum yield and distributions that would indicate protein or dye degradation. The pooled data from all three proteins were compared for similar anomalies that would occur due to mutation/labeling. The data presented in the ensemble and time-dependent analyses were free of any such anomalies.

General Data and Wavelet Analysis

After multiply labeled proteins have been removed from the analytical sample, each trajectory undergoes automated photoblink removal by Bayesian statistical analysis. Each trajectory is then analyzed to confirm that FRET is occurring between two dyes that are unhampered by photophysical interactions with the protein host. This is accomplished by comparing donor photon counts, acceptor photon counts, total photon counts, and background counts for each trajectory as compared to the ensemble average. Additionally, donor and acceptor autocorrelation analysis is performed for each trajectory to ensure that the dyes are not rotationally hindered. Finally, donor-acceptor cross-correlation analysis is performed to check that the photon count response is anti-correlated for each trajectory. What results, in the case of a donor/acceptor-labeled biomolecule with a static conformation is a trajectory that is up to 97%
free of photoblinks and broadened only by shot-noise. Such a trace is illustrated in Figure 2 of
the manuscript, along with a histogram of the smFRET occupancy percentage for the trajectory
before and after denoising.

More complicated smFRET trajectories, in which the molecule undergoes a
conformational change, result in dynamic changes in the smFRET values, and also in the relative
shot-noise in that trace (because shot noise is a function of the total amount of signal on each
detector). Such a trace is shown in Figure 2d. After blink removal, the donor and acceptor
photon trajectories are first transformed from the time domain to separate, orthogonal
components in the wavelet domain. This is accomplished via convolution of each photon
trajectory with low pass and high pass digital filters, and is similar to Fourier filtering in its
theory but is more appropriate for the finite-time domain data collection in single molecule
analysis2-3. The component generated by the low pass filter contains low frequency information
about the signal, and high frequency information is contained within the component generated by
the high pass filter. The magnitude of the shot-noise fluctuation in each signal is used to
generate a threshold, and this threshold is used to remove the shot-noise contribution from the
high frequency component via the procedure described in our earlier work2-3. These denoised
wavelet components are used to reconstruct the photon trajectories by inversion of the wavelet
transformation. This procedure reduces the contribution of shot-noise to the data, thereby
increasing the resolution of the experimental data.

Sample raw and denoised donor and acceptor photon trajectories for single glutamate-
bound ABD proteins are shown in Figures 2A and D. In 2B and E, the calculated apparent
smFRET trajectory is shown along with the denoised version. Also shown are the results of
state-finding analysis on the denoised data by Hidden Markov Method4. As will be further
discussed below, accurate state-finding on multi-state and broadened smFRET trajectories is
made possible by first using wavelet denoising on the trajectories. Figures 2C and F show
smFRET histograms generated from the raw data, the denoised data, and the HMM analysis of
denoised data.

II. Supplementary Results

Denoised Ensemble smFRET Histograms

The utility of analyzing denoised smFRET data was demonstrated in our earlier work2
and recently optimized3 for complex multi-state systems such as the AMPA receptor. In
particular, when we examine ensemble smFRET histograms of a system comprised of close-
lying states in equilibrium with each other, the shot noise obscures our ability to resolve these
systems, as shown in Figure S5A. When we use wavelet techniques to reduce the experimental
standard deviation about the underlying states, as is shown in Figure S5B, it is possible to more
accurately identify those states, their distributions, and the rate constants between those states, as
is shown in Figures S5C and D.
Figure S5. (A) Ensemble smFRET histogram of a 2-state equilibrium with states centered at 0.81 and 0.89. (B) Histogram after wavelet denoising. (C) State-finding analysis on raw data and (D) on denoised data demonstrates that the standard deviation due to shot-noise around the states can be reduced by denoising, resulting in more accurate extraction of states, distributions, and rate constants."
Figure S6 includes the raw and denoised ensemble smFRET histograms for all three GluR2-ABD protein systems studied.

**Figure S6.** Observed and Denoised Ensemble FRET Histograms. (A) Observed apo GluR2-ABD, 41 molecules. (B) Denoised apo GluR2-ABD. (C) Observed Glutamate-bound GluR2-ABD, 67 molecules. (D) Denoised Glutamate-bound GluR2-ABD. (E) Observed Glutamate-bound GluR2-ABD-T686S, 60 molecules. (F) Denoised Glutamate-bound GluR2-ABD-T686S.
Equilibrium State and Transition Lifetime Analysis

It is important to contrast the various ways that states could be identified in a distribution of values. Most commonly, a histogram such as any depicted in Figure S6 could be fit with multiple Gaussian curves. This would result in highly accurate fits and determinations of the likely FRET values that contribute to the ensemble distribution, but would have a questionable relationship to any underlying physical states, and is therefore of little value in determining conformational landscapes.

The Hidden Markov Algorithm used in our state-finding analysis is based on a Markovian dynamics for kinetic transitions of EACH smFRET trajectory, and relates state values and rate constants for all transitions\(^5\). As we have previously shown\(^2\) and discuss below, performing such analysis on denoised smFRET trajectories allows accurate extraction of states AND rate constants for multi-state processes that are otherwise irresolvable.

After state-finding was performed on each trajectory, a separate dwell-time analysis (described further below) was performed for each state-to-state transition, as discussed and depicted in Figure 4 of the main text. In this analysis comes the first evidence for sequential equilibrium in addition to the previous theoretical examination\(^5\). As is clearly shown in Figure 4 of the main text, for every state, transitions are almost exclusively determined to occur to a neighboring state. This can be further demonstrated when we examine transitions between initial and final smFRET values for every observed transition in each single glutamate-bound ABD protein, as shown in Figure S7. Thus, even without identifying states, the data support transitions to distinct, adjacent smFRET domains.
The transition lifetime data for the glutamate bound, apo, and T686S forms of the GluR2-ABD analysis are included in Tables S2, S3, and S4, respectively.

Table S2. Apo GluR2-ABD State Transitions

| Equilibrium | Transition | Initial Population | Net Transition |
|-------------|------------|--------------------|---------------|
| 0.54 ↔ 0.65 | 0.54 → 0.65 | 41                 | 0.54 → 0.65   |
|             | 0.65 → 0.54 | 38                 |               |
| 0.65 ↔ 0.75 | 0.65 → 0.75 | 36                 | 0.65 → 0.75   |
|             | 0.75 → 0.65 | 33                 |               |
| 0.75 ↔ 0.84 | 0.75 → 0.84 | 28                 | 0.75 → 0.84   |
|             | 0.84 → 0.75 | 20                 |               |
| 0.84 ↔ 0.94 | 0.84 → 0.94 | 17                 | 0.84 → 0.94   |
|             | 0.94 → 0.84 | 13                 |               |

Table S3. Glutamate-bound GluR2-ABD State Transitions

| Equilibrium | Transition | Initial Population | Net Transition |
|-------------|------------|--------------------|---------------|
| 0.59 ↔ 0.72 | 0.59 → 0.72 | 43                 | 0.59 → 0.72   |
|             | 0.72 → 0.59 | 34                 |               |
| 0.72 ↔ 0.81 | 0.72 → 0.81 | 51                 | 0.72 → 0.81   |
|             | 0.81 → 0.72 | 46                 |               |
| 0.81 ↔ 0.90 | 0.81 → 0.90 | 41                 | 0.81 → 0.90   |
|             | 0.90 → 0.81 | 40                 |               |

Table S4. Glutamate-bound GluR2-ABD-T686S State Transitions

| Equilibrium | Transition | Initial Population | Net Transition |
|-------------|------------|--------------------|---------------|
| 0.39 ↔ 0.54 | 0.39 → 0.54 | 24                 |               |
|             | 0.54 → 0.39 | 24                 |               |
| 0.54 ↔ 0.68 | 0.54 → 0.68 | 44                 | 0.54 → 0.68   |
|             | 0.68 → 0.54 | 42                 |               |
| 0.68 ↔ 0.82 | 0.68 → 0.82 | 49                 |               |
|             | 0.82 → 0.68 | 49                 |               |
| 0.82 ↔ 0.96 | 0.82 → 0.96 | 36                 | 0.82 → 0.96   |
|             | 0.96 → 0.82 | 32                 |               |
Simulated 4-State Equilibrium

Figure S8. Denoising a simulated 4 state system. A) shows the relative occupation of each of the 4 simulated states. Adding a zero-mean, Gaussian white noise to each of the acceptor and donor trajectories, subsequent background and crosstalk correction, and calculation of efficiencies leads to the distribution shown in B). The denoised complement to B) is shown in C). Applying the HMM to the raw and denoised trajectories results in the distributions shown in D), and E), respectively. Parameters extracted from the hidden-Markov modeled trajectories are reported in F).

To demonstrate the ability to extract accurate information from a noisy efficiency distribution obtained from a complicated, multi-state system, we use Kinetic Monte Carlo (KMC) methods to simulate the system shown in Fig. S8. The KMC-simulated trajectories consist of 4 states having central efficiencies 0.9, 0.8, 0.7, and 0.6, and each state has an escape rate in the range of 4.5 – 14 Hz, corresponding to average lifetimes in the range of 70 – 200 ms. The relative occupation of each state is shown in Fig. S8A. State trajectories are simulated first, with acceptor and donor photon trajectories being constructed from the state trajectories. The sum of acceptor and donor photon counts has a mean of 185 photons per time step for all of the 14,000 time steps in 35 simulated trajectories. The signal to background ratio is 2.3, and the donor to acceptor background ratio is 2. The crosstalk ratio was fixed at 10 %. Shot-noise was simulated at the \( i \)th time step as an additive, zero-mean, Gaussian white noise component with magnitude

\[
\sigma_A(i) = \sqrt{N_A(i)} \quad \text{for the acceptor signal, and} \quad \sigma_D(i) = \sqrt{N_D(i)} \quad \text{for the donor signal.}
\]

Here, each of the \( \sigma_A(i) \) and \( \sigma_D(i) \) represent standard deviation, and each is independently and identically distributed for all time steps. After correction for background and crosstalk, efficiencies are calculated and compiled to generate the distribution shown in Fig S8B. Each of the 35 acceptor and donor trajectories is then denoised via the procedure described in our published work, and, after similar background and crosstalk correction, denoised efficiencies are calculated and compiled to generate the denoised efficiency distribution shown in Fig. S8C.
Hidden-Markov model (HMM) analysis \(^4\) is then used to identify states in each of the noisy and denoised efficiency trajectories. So that the same central efficiencies are identified in each trajectory, efficiency trajectories are concatenated, and the HMM is instructed to find the most likely states in the trajectories. The relative occupation of the states extracted by the HMM from the noisy data is shown in Fig. S8D, and that extracted from the denoised data is shown in Fig. S8E. Fig. S8F tabulates the information extracted by the HMM analyses. The escape rates shown in Fig. S8F are extracted by dwell time analyses, in which the distribution of each state’s dwell times is fit to an exponential decay.

The comparison shown in Fig. S8 demonstrates that, although the distribution of shot-noise-induced efficiencies of our model system has unremarkable features and indistinguishable states, denoising the trajectories with the procedure described in Ref. \(^2\) allows for the accurate extraction of information about the relative occupation of each state, of each state’s central efficiency, and of the kinetic aspects of the system. Inspection of the distribution shown in Fig. S8D – the relative occupations of each state extracted from the noisy data by the HMM – reveals that the state having central efficiency 0.7 is not identified by the HMM. This is due to the broadening effect of the shot-noise contribution. Furthermore, because of shot-noise broadening and the unidentified state, the kinetics extracted by the HMM from the noisy data are skewed and unreliable at best. In contrast, the central efficiencies extracted from the denoised data have negligible deviation from their true values, relative occupations have average deviation of < 5 \%, and the extracted escape rates have an average deviation of only 8.5 \%. The precision and accuracy of the system properties extracted after denoising therefore demonstrates the remarkable enhancement to our ability to extract accurate information from complicated efficiency distributions via the application of the denoising procedure\(^2\).

Dwell Time Analysis

Dwell time distributions are compiled by probabilistically assigning states to each time step in each trajectory of each model system, and extracting dwell times in each state (once the states have been identified using a Hidden Markov method \(^4\) on the wavelet-denoised data \(^2\)). The resulting distributions are fit with a single exponential decay whose exponential coefficient is the rate constant for escape from that particular state. This is demonstrated most easily for a simple two-state equilibrium. KMC methods described above were used to simulate smFRET trajectories for a two-state equilibrium with shot noise added. After denoising and HMM state-finding analysis, dwell times were extracted for each of the two identified states. Fig. S9A shows the

![Figure S9. Dwell time analysis. A) shows the dwell time distribution of the 0.9 efficiency state in the two state system fit to an exponential decay. B) shows that of the 0.4 efficiency state.](image_url)
dwell time analysis for the 0.9 efficiency state of the two state model system, and Fig. S9B shows that of the 0.4 efficiency state.

This analysis gets a step more complicated when there are multiple states to which a transition can occur. In this case, a further separation is performed in order to extract dwell times from a particular state to another particular state, but in essence, the analysis is identical to that demonstrated above. The data for this analysis on the glutamate-bound GluR2-ABD smFRET trajectories after state-finding analysis was performed for the 12 possible transitions among the 4 states is shown in Figure 4 of the main text. The dwell times for each possible transition were fit to single exponential decays, as was shown in Figure S9. Figure S10 illustrates the denoised ensemble smFRET histogram for the glutamate-bound GluR2-ABD form, with states and rate constants assigned.

The results demonstrate several important points. First, there are statistically insignificant transitions to non-adjacent states, confirming sequential equilibria. Next, except for the transitions into/out of the most closed form, the decays are well-fit to single exponential processes. This suggests that the time scale of the measured events are well described by single exponential kinetics that occur within the time resolution of our measurements.

To confirm this, kinetic Monte Carlo simulations were performed for sequential 4-state equilibria with rate constants matching those fit to our experimental data, and with rate constants ten and one thousand times faster, as might be expected if our measurements were monitoring binding/dissociation events. The resulting transitions were incorporated into simulated smFRET histograms and are compared with the actual denoised data in Figure S11. This comparison makes is clear that it is not possible we are measuring time-averaged snapshots of faster events. Only the smFRET histogram compiled from transitions with similar rate constants accurately simulates the actual data. It is interesting to note that the only state that is less accurately simulated is the most closed state, which might be associated with a faster docking transition. In this case, the smFRET dwell times would be expected to occur via a convolution between faster docking events and slower conformational transitions, and the data bear this out.
A more extensive rate analysis was performed to determine the expected accuracy of experimentally determined transition rate constants for transitions that occur faster than our observation window. The results of this analysis are shown in Figure S12. Here it can be seen that in a two-state transition, the bimodal distribution of states gradually collapses into a single, broadened state as the transition frequency approaches the sampling frequency. Thus, although it is possible that our measurements detect events on the order of 10 times faster than our sampling resolution, the broadened smFRET distributions, because they are compiled from single molecule traces with a 1 ms sampling frequency, are dominated by events that occur on or slower than the 1 ms time scale. Faster events would simply be averaged out, and this is akin to taking an ensemble-averaged FRET measurement.

**Figure S11.** Histograms of ensemble smFRET values from experimental GluR2-ABD data (dark blue) are compared to those compiled from simulated data with rate constants on the same order of magnitude, ten times, and 100 times faster than the extracted values, respectively. Only the simulated data with rate constants on the same order of magnitude as the fitted data approximate our observed histogram.

**Figure S12.** Resolution of two states in equilibrium is dependent on the rate of transition between the two states. A) The bimodal efficiency distribution of simulated two-state systems is shown to collapse to a unimodal distribution as the transition frequency approaches and exceeds the sampling frequency. B) The standard deviation of each distribution as a function of transition frequency between the two states in the simulated equilibrium.
Finally, the rate constants presented in the text (for the glutamate-bound protein) were used in kinetic Monte Carlo simulations to assess their ability to recreate the single-molecule ensemble data. The experimental histogram of the glutamate-bound GluR2-ABD shown in Figure S13A compares favorably with the histogram in Figure S13B, which was simulated with the rate constants extracted using dwell-time analysis as presented in Figure 4 of the main text.

**Figure S13.** Ensemble smFRET histograms for glutamate-bound GluR2-ABD. A) Experimental data. B) Simulated data using rate constants extracted from dwell-analyses (Figure 4 in main text).
Supplemental References

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