Supporting Information

Blunck *et al.* 10.1073/pnas.0807056106

SI Text

Robustness of Fit. The models (Fig. 4 *C* and *D*) have 15 and 31 states, respectively. By fixing k_B to the overall bleaching rate (Table S1) and running different fits for each coupling constant C, we could reduce the number of free parameters to only 2. We wanted to know how reliably the algorithm would find the correct rate constants of the model. Therefore, we simulated time traces based on the dependent model (the independent model always gave exactly the same results as the dependent model with C = 1) with different combinations of the 4 parameters. We subsequently fitted the traces with the model by using different starting values. For each combination of α and β, five traces were simulated and fitted with high as well as low starting values. Starting values were chosen at both extremes, too high and too low. Fig. S2 shows the results of the fitting of the simulated data as a function of the modulated parameter α and β or the coupling constant C. In general, the transition rate constants corresponded well to the theoretical values (solid lines) for the different combinations of parameters. The only problem occurred if α was close to the bleaching rate k_B. In this case, a low starting value for β either tended toward zero or remained close to its starting value. If there was a high starting value for β, the correct values were found. In our actual data, this could only occur at pH 6–7, where the opening rate constant is very low. This may explain why we could not fit many of the data at pH 7 (open probability too low). In all other cases (lower pH), α is larger than k_B. In the fits of the actual data, we used high and low starting values and ensured that the fits converged to identical results.

If α and β were higher (e.g., = 1), the rate constants estimated by the Markov model were determined slightly too low. (Fig. S2*a,* green symbols). However, the ratio between α and β remained correct, so that the open probability calculated from the rate constants gave the correct value (Fig. S2*c*).

Calculation of Double-Jump Probability

Assumptions:

1. We consider 2 identical systems which behave according to a Markov system.
2. Each system may reside in 2 states.
3. α and β are the closing and opening rate constants.
4. The temporal resolution is Δt = 30 ms.

The probability of the system to reside in the all closed state is:

$$P_{CC}=\left(\frac{\beta}{\alpha+\beta}\right)^2 \quad [1]$$

We wait for 1 of the 2 systems to open and calculate the probability of the second one to open within the time Δt after this event (Fig. S1).

The probability that a closed system will open within a certain interval Δt can be calculated by integration of the dwell time histogram (normalized to the integral to infinity under the histogram) and is equal to:

$$P(\alpha,\Delta t) = (1 - e^{-\alpha\Delta t}) \quad [2]$$

At the same time, the first system must not reclose:

$$P_{RO}(\beta,\Delta t) = e^{-\beta\Delta t} \quad [3]$$

Thus, the probability of a system residing in state CC to open in a double step is:

$$P_{DSC}(\alpha,\beta,\Delta t) = e^{-\beta\Delta t}(1 - e^{-\alpha\Delta t}) \quad [4]$$

The probability for a double opening to occur in an equilibrated system is thus:

$$\mathrm{PDC}=P_{DSC}(\alpha,\beta,\Delta t)P_{CC} = \left(\frac{\beta}{\alpha+\beta}\right)^2 e^{-\beta\Delta t}(1-e^{-\alpha\Delta t}) \quad [5]$$

The closing probabilities (P_{DSO}, PDO) may be calculated accordingly.

The probability for a double opening and subsequent closing is then:

$$\mathrm{PDCO}=P_{DSC}(\alpha,\beta,\Delta t)P_{DSO}(\alpha,\beta,\Delta t)P_{CC} \quad [6]$$

and for a double closing and subsequent opening:

$$PDOC = P_{DSC}(\alpha,\beta,\Delta t)P_{DSO}(\alpha,\beta,\Delta t)P_{OO} \quad [7]$$

The probability for a double event of either direction to occur is thus:

$$PD = P_{DSC}(\alpha,\beta,\Delta t)P_{DSO}(\alpha,\beta,\Delta t)(P_{OO} + P_{CC}) \quad [8]$$

(Because the dwell times are supposed to be long compared with Δt, we did not consider the case that the second system opens but closes before the end of Δt.)

Results. The probability for a double step of 2 subunits (PDC/PDO) and for a double event in either direction (PD) have been calculated by using an exposure time Δt = 0.03 s and dwell times varying between 100 ms and 20 s.

For the dwell times that we observed in our experiments, the PDC_{exp} was <1%. The highest probability in the range for a double step was PDC_{max} = 4.8%. This was for the shortest dwell times of 100 ms for both opening and closing. However, much shorter dwell times do not further increase the percentage of double steps, because the probability of the first system to reclose would be too large. The optimal values are thus a function of the sampling interval Δt. The maximal probability of 6.7% was reached at 61 ms and 39 ms for the opening and closing dwell time, respectively. With dwell times so close (or even below) our exposure time, we would not clearly resolve the signals anymore but rather increase the noise.

The probability of observing a double closing and opening step is even lower. All values similar to the experimental values gave probabilities of well below 1% (PD_{exp} < 1%). The maximum is 3.1% at dwell times of 50 ms and 38 ms for opening and closing, respectively.

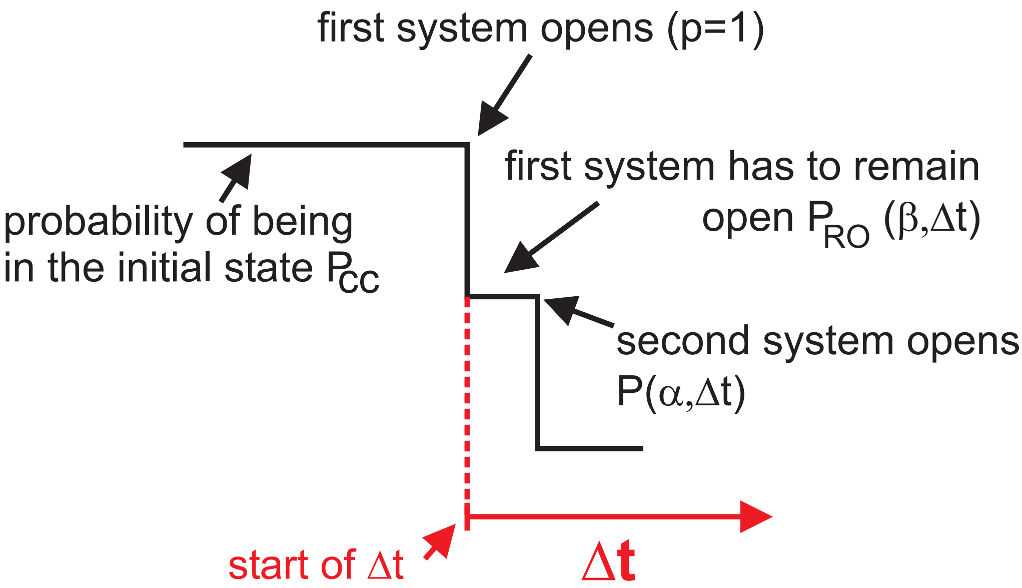

Fig. S1. Calculation of double-jump probabilities

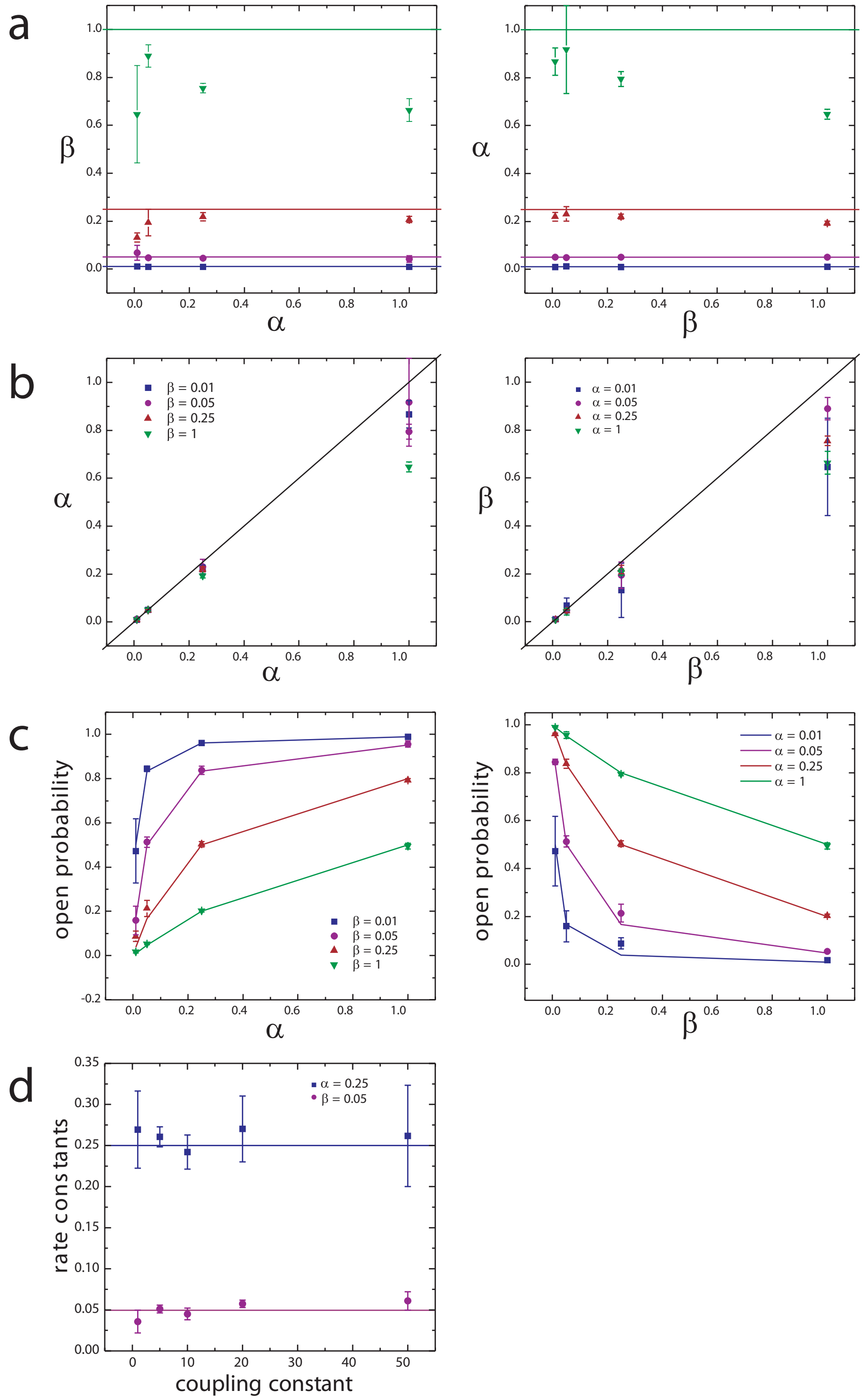

Fig. S2. Robustness of fit. Results of fitting simulated data with the dependent model. On the abscissa, the theoretical value used to simulate the data, on the ordinate the mean and standard deviation of the fits of 5 simulations are shown. The lines indicate the theoretical values. For each point 5 time traces were simulated and fitted with high and low starting values. Open probability was calculated as $\alpha/(\alpha + \beta)$ by using the optimal fit results.

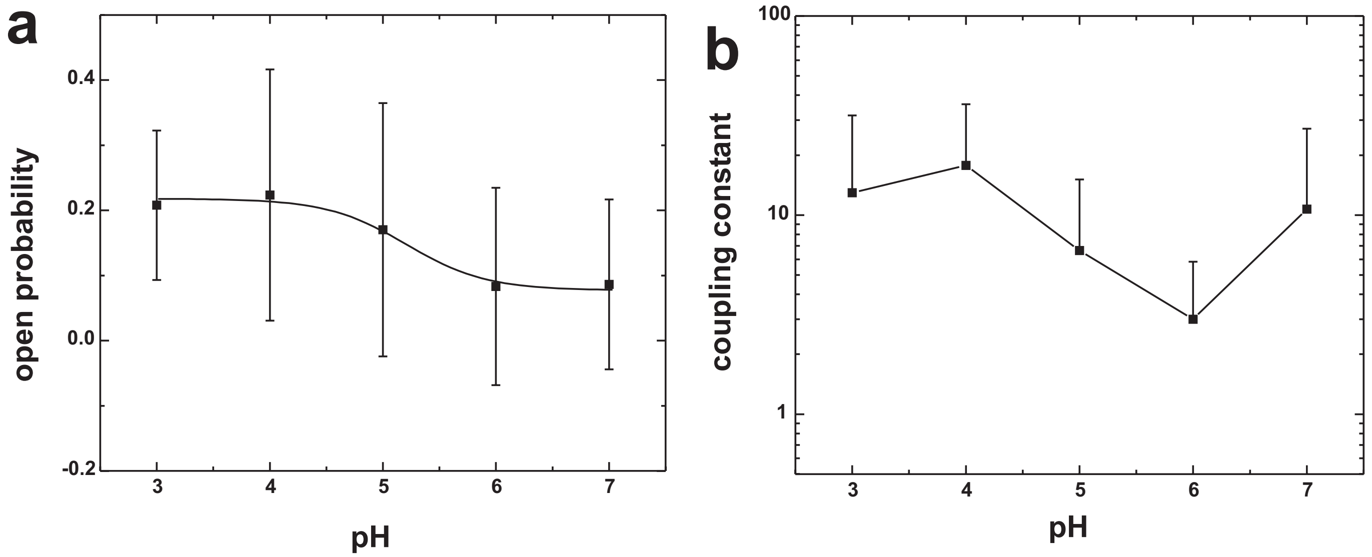

Fig. S3. Results from ATTO-565M imaging. (a) Open probability of one subunit of Q119C-E71A determined directly from the fluorescence traces labeled with ATTO-565M. The data are fitted to a Boltzmann curve with pK = 5.2. (*b*) Coupling constant (mean and SD*;* neg. SD omitted in logarithmic scale for clarity) as a function of Q119C-E71A labeled with ATTO-565M.

Table S1. Comparison of bleaching rates from exponential fit and QUB

Image	k_B	$\langle k_B \rangle$	k_τ
1	0.014	0.030 (0.036)	0.029
	0.0000017		
	0.055		
	0.014		
	0.065		
	0.030		
2	0.025	0.019	0.029
	0.012		
3	*0.00405*	0.022 (0.031)	0.037
	0.024		
	0.037		
4	0.031	0.026 (0.033)	0.031
	0.0063		
	0.034		
	0.034		
5	0.016	0.011 (0.017)	0.017
	0.017		
	0.0000022		

Bleaching rates for 5 different images are shown. Several single spots per image were fitted with the Markov models (Fig. 4) in QUB with free bleaching rate k_B (2nd column). $\langle k_B \rangle$ is the averaged bleaching rate of these spots. The values were compared with the bleaching rates k_τ obtained directly from exponential fitting of the integrated intensity of the entire image (4th column). Not all values fit perfectly (see *italics*), which is not surprising, because we have a maximum of 4 events to determine the rate. $\langle k_B \rangle$ values in parentheses are calculated excluding the values in *italics*.