Electronic Supporting Information

"Quantitative Detection of Conformational Transitions in a Calcium Sensor Protein by Surface Plasmon Resonance"

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Supporting Methods and Materials

Preparation of protein samples, buffers and calcium stocks

Recombinant mRec and its mutant $mRec^{E121Q}$ were prepared as described previously (See Ref. [4] in the main text).

For SPR and ITC experiments, the buffer in use (5 mM Tris/HCl pH 7.5, 150 mM KCl) was applied on a Chelex 100 resin column (Bio-rad) with a flow rate of 3 mL/min to remove Ca^{2+}-contaminants. Before use the Chelex resin was activated by incubation with 6 M HCl for 10 min, washed on a filter (grade 3hw) with 1 L of H_2O_{bidest}, incubated with 2M KOH for 10 minutes, then washed again with 1 L of H_2O_{bidest}. The pH of the slurry in 100 mL H_2O_{bidest} was adjusted between 7 and 8 with 2 M HCl. Finally, the resin was incubated for one day with the Tris buffer (s- above) before the final pH adjustment was made. The remaining concentration of Ca^{2+} in the buffer after decalcification was measured by withdrawing a small volume and by performing a BAPTA absorption assay as described.[1] It was found to range between 0.11 and 0.20 µM.

$CaCl_2$ of the highest grade available was dissolved in the decalcified buffer to a final 46 mM concentration and Ca^{2+} stocks were prepared by subsequent dilutions to final concentrations used in SPR experiments (injections in Figure 1): 0.4 µM, 0.7 µM, 0.9 µM, 1.1 µM, 1.6 µM, 2.5 µM, 4.8 µM, 14 µM, 37 µM, 46.2 µM, 92.2 µM, 0.46 mM, 0.92 mM and 2.3 mM. Further 0.5-1 mM Ca^{2+} stocks were prepared for ITC experiments. All buffers were filtered (0.22µm) and degassed for at least 1h before use. For SPR runs Tween20 was added at a final concentration of 0.005% (v/v).

We noticed that Ca^{2+} buffers prepared from stock solutions of K_2H_2-EGTA and $K_2CaEGTA$ as described by Tsien and Pozzan[2] were not suitable in our particular

application of the SPR technology. This was likely due to multiple equilibria involving EGTA-Ca^{2+}-protein interactions leading probably to effects of EGTA on the refractive index. These effects might have covered the modest changes due to the conformational transitions (results not shown). Therefore the decalcified Tris buffer was chosen instead for all the experiments.

Surface Plasmon Resonance experiments

A Biacore2000 surface plasmon resonance instrument (GE Healthcare) was employed for detecting conformational changes in mRec upon injections of Ca^{2+}. A 10 mM HEPES pH 7.4, 0.15M NaCl, 0.005% Tween20, 3 mM EDTA buffer was used as a running buffer for the immobilization of protein samples at T = 25ºC. Commercially available (GE Healthcare) sensor chips CM5 (coated by a carboxymethyldextran (CMD) layer that extends about 100 nm over a monolayer of selfassembled alkanethiols on the chip gold surface) were used for all experiments. CMD matrix-free sensor chips C1 (GE Healthcare) with only a flat carboxylated surface to cover the gold surface were also tested in our experiments, resulting in much lower levels of protein immobilization, very small signals upon Ca^{2+} injections and rapid protein degradation (results not shown).

Ligand thiol coupling was performed as described before for mRec by making use of the single cysteine at position 38.[3] Lyophilized proteins dissolved in water were diluted into 10 mM sodium-acetate buffer pH 4.2 to a final concentration of ~25 μg/mL and flowed for 7 min on the sensor chip, thus leading to an immobilized amount of about 6 $ng \times mm^{-2}$ for each protein. We determined the protein concentration within the dextran matrix by assuming a volume of ~0.02 μL for each flow cell (from the instrument manual). One of

the four flow cells in the sensor chip was always reserved for a blank immobilization with no protein in the coupling step, and was used as a reference. The other flow cells were used for immobilization of mRec and mRec^{E121Q} at similar final levels. Before each replicate of Ca^{2+} titration experiments, the stability of the immobilized proteins was verified by recording a stable baseline for 30-60 min in the presence of decalcified running buffer only. Immobilized proteins were found to be sufficiently stable for no longer than 2-3 days, after which the overall signal and quality of sensorgrams were significantly compromised.

In order to check for potential effects of the orientation of the immobilized proteins on the observed phenomenon, we performed the same Ca^{2+} titration experiments with mRec and mRec^{E121Q} immobilized on the sensor chip via the non-site-specific amine coupling chemistry. We followed an established procedure described earlier[3] while starting from the same initial protein stocks and immobilization buffers as above. The immobilized level of mRec (~7330 RU) was roughly 2000 RU higher compared to the one obtained via ligand thiol coupling, why that of mRec^{E121Q} was ~2000 RU lower. Results of such control experiments are reported in Figure S2. Experiments at higher Ca^{2+} concentration in the mM range were performed also in this case, resulting in very similar trends compared to those reported in Figure 1e (results not shown).

Analysis of SPR experiments

The Ca^{2+} binding capacity (RU_{max}) was calculated for each flow cell with immobilized protein according to the formula:

$$RU_{\max} = \frac{MW_{Ca^{2+}}}{MW_{mRec}} RU_{mRec}^{imm} \, n_{Ca} \qquad (1)$$

in which the atomic mass of Ca^{2+} (40 Da) and the molecular mass of the protein (23 kDa) are accounted together with the number of possible binding sites (n_{Ca}=2 for mRec and 1 for $mRec^{E121Q}$) and the amount of protein immobilized in the flow cell, in RU units (1 RU corresponds to 1 pg protein mm^{-2}).

Titrations with Ca^{2+} were performed by injecting 20 µL of each Ca^{2+} stock with a flow rate of 10 µL/min. The maximum amplitude after each injection was recorded for the equilibrium analysis. Injections were followed by flow of decalcified buffer only, and a time frame of ~140 s preceded the following injection. Each injection therefore corresponded to 120 s association/transition and 120 s dissociation/relaxation followed by a ~20 s restore time before the next injection. Titrations were repeated four times with fresh Ca^{2+} stocks in capped plastic vials to minimize solvent evaporation. The maximum amplitudes upon Ca^{2+} injection were averaged and the standard deviations were calculated. The resulting curve (Fig. 1c) was fitted according to the Hill sigmoid :

$$RU = RU_0 + \frac{\Delta RU}{1 + \left(\frac{[Ca^{2+}]}{K_D^{app}} \right)^h} \qquad (2)$$

in which ΔRU is the difference between the maximum RU and the baseline (RU_0), h is the Hill coefficient and K_D^{app} is the [Ca^{2+}] at which the response is half-maximal.

Kinetic analyses were performed on 11 injections, 9 of which corresponded to 1.6, 2.5 and 4.8 µM [Ca^{2+}] injections from three independent experiments described above. In addition, two longer experiments (240 s Ca^{2+}-injection, 240 s dissociation/relaxation) were performed at 2.5 µM and 14 µM [Ca^{2+}] and the results were statistically analyzed together with the other data. For the analysis, a simple model for the transition T to R was employed, and the dissociation/relaxation phase was analyzed first:

$$R \xrightarrow{k_{off}} T \quad (3)$$

which could be fitted by a simple exponential decrease of SPR signal resulting from the first-order ordinary differential equation to give k^{off}. The off-rate constant was then used as a fixed parameter for fitting the association/transition phase according to a pseudo-first order scheme:

$$T \xrightarrow{k_{on}^{app}} R \quad (4)$$

in which $k_{on}{}^{app} = k_{on}[Ca^{2+}]$. During the injection phase, concurrent association and dissociation (and concerted structural transition) processes may occur to give a SPR signal that is fitted by the following solution of the corresponding pseudo first-order ordinary differential equation:

$$RU(t) = R_0 + \frac{k_{on}^{app} R_{\max}}{k_{on}^{app} + k_{off}} \left(1 - \exp(-(k_{on}^{app} + k_{off})\, t\right) \quad (5)$$

in which R_0 is the baseline value, $R_{\max}$ indicates the maximum SPR response that would be expected if all immobilized and active mRec would undergo the Ca^{2+}-induced transition and k_{off} is the off-rate constant obtained in the independent fitting analysis of the dissociation phase of the same injection.

Slight time delays in controlling and switching valves in the microfluidic system of SPR devices can lead to distortions in SPR sensorgrams for the first seconds after each injection and dissociation (Figure S1). Therefore, the points recorded in the first 4-10 s after each injection and dissociation were omitted by the fitting procedures.

From each kinetic analysis, k_{on} and k_{off} values were obtained and the average and the standard deviation for each parameter were calculated. The average equilibrium constant and the relative free energy were given according to the relationship:

$$\Delta G = -RT \ln K_{eq} = -RT \ln \left(\frac{k_{on}}{k_{off}} \right) \quad (6)$$

All the fittings concerning SPR data were performed with Kaleidagraph 3.51 (Synergy software).

Isothermal Titration Calorimetry experiments

ITC experiments were performed using a VP-ITC instrument form MicroCal (Northhampton, MA) at T=25ºC. The same buffer was used in the recording and the reference cell. All solutions were thoroughly degassed by stirring under vacuum before use. Decalcified mRec was dissolved in decalcified Tris-buffer at 19-25 μM and was placed in the recording cell (1.46 mL). The concentration of mRec was checked by absorption at λ = 280 nm using an extinction coefficient of 32,200 $M^{-1}cm^{-1}$. $CaCl_2$ prepared in the same buffer was placed in the microsyringe chamber at 0.5-1 mM. Ca^{2+} titration were performed by 45-50 subsequent injections of 5 μl Ca^{2+} -solution into the recording cell keeping a time-interval of 180 s between injections and an initial delay of 600 s after temperature equilibration. Control injections of Ca^{2+} into the decalcified buffer without protein were also performed, but did not result in specific heat changes. The baselines of the raw data were automatically adjusted by Origin (MicroCal) and the same software was employed for data analysis and fitting. Optimal fitting results were obtained with the "one set of sites model", providing an estimate for number of binding sites, K_D^{app} and molar enthalpy (ΔH) and entropy (ΔS) changes at the given temperature. Some ITC experiments were performed also with 50mM HEPES/KOH pH 7.5, 100mM KCl buffer at T = 25ºC and 30ºC, resulting in quantitatively very similar results. Overall,

the K_D^{app} values obtained by fitting in 8 independent experiments were averaged and the standard deviation calculated. One example of ITC titration curve is reported in Figure 2.

Estimation of Ca^{2+}-occupancy levels in mRec

The pure binding of Ca^{2+} to the myristoylated form of recoverin has been shown to occur with cooperativity and with an apparent K_D = 17-18 μM (see Refs.[1,2] in the Main Text). It is however possible to separate the individual contributions of each EF-hand (EF3 and EF2) to Ca^{2+}-binding according to an allosteric model, leading to different affinities for the two binding sites (K_D^{EF3} = 0.11 μM and K_D^{EF2} = 6.9 μM) (see Ref.[1] in the Main Text). If such individual binding constants are considered and knowing the initial concentration of mRec ($mRec_{tot}=mRec_{free}+mRec\cdot Ca$) in our SPR experiments, the occupancy of each individual binding site at the [Ca^{2+}] corresponding to half-maximal transition can be calculated, for instance for EF3, from the relationship:

$$K_D^{EF3} = \frac{\left[mRec_{free}\right]\left[Ca^{2+}\right]}{\left[mRec \cdot Ca\right]} \qquad (7)$$

in which [Ca^{2+}] is replaced by K_D^{app} from SPR experiment, and the occupancy level can be therefore calculated as the ratio between Ca^{2+}-bound and total mRec. The same procedure applies to EF2.

Structural analysis

Data from NMR structural assignments were employed to described the T (PDB entry: 1IKU) and the R (PDB entry: 1JSA) conformations of mRec. Average structures and solvent-accessible surfaces were calculated by using QUANTA2005 (Accelrys). The

radius of gyration for each conformation was calculated using WORDOM[4] starting from the coordinates of each average structure as an input. After removal of hydrogen atoms, the molecules were graphically represented by using PYMOL 0.97 (http://pymol.sourceforge.net/

Fig. S1 Raw data from SPR experiments: comparison between Ca^{2+} injections on the reference cell and the protein-coated cells. The same 240 s injection of 2.5 μM Ca^{2+} was performed on both the reference cell, without immobilized protein (dashed line) and the cell in which 12.7 μM mRec was immobilized (solid line). The switching valves in the microfluidic system and the slight delay in the flow cells path induced an abrupt distortion of the sensorgram in the very first seconds (4-10 s) of both the rising and decreasing phases, when the injected solution is changed. Such time frames have been therefore excluded by the fitting procedure.

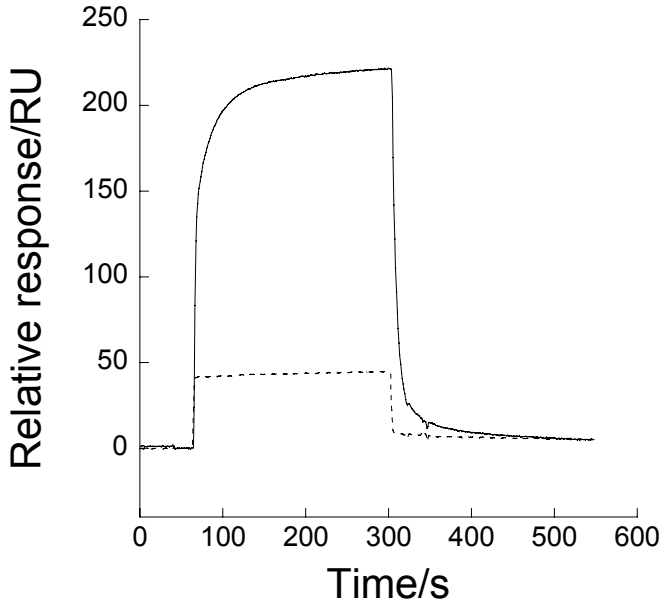
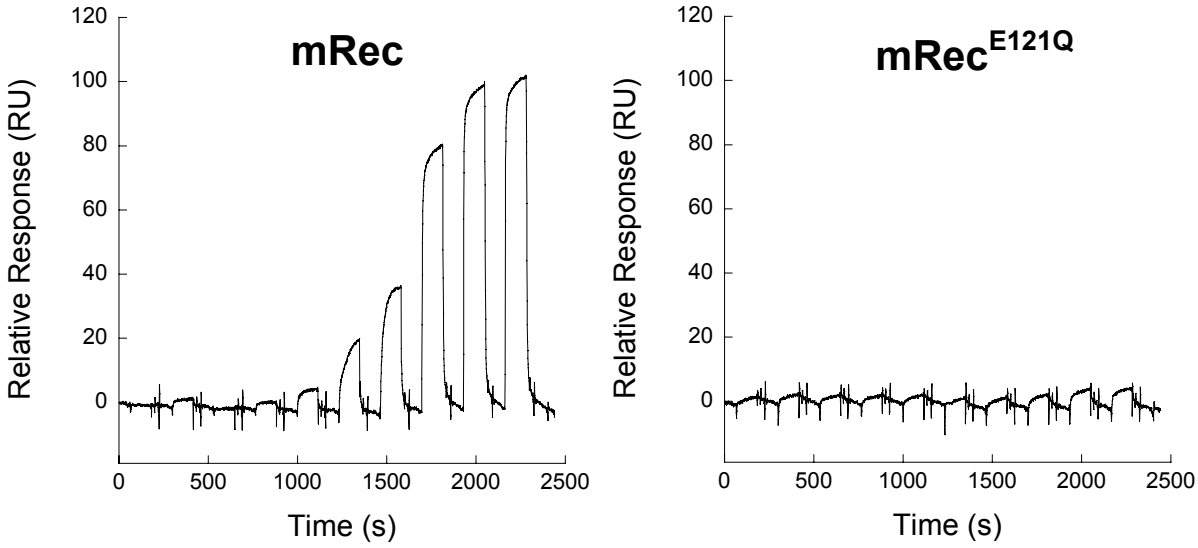


Fig. S2 SPR detection of Ca^{2+}-induced conformational changes in mRec (left) immobilized on the sensor chip via non-site-specific amine coupling. The same titrations were performed with the $mRec^{E121Q}$ variant (right) and the Ca^{2+} concentrations were very similar to those in Figure 1a and 1b. Experiments were repeated to ensure reproducibility. The same trend and a good quantitative agreement with the data obtained via ligand thiol coupling (K_D^{app} ~6 μM) suggest the method is only partially sensitive to the orientation of the immobilized molecules within the dextran matrix and it is suitable to other immobilization startegies. However, despite the higher immobilization level obtained for mRec via amine coupling, the intensity of the phenomenon in this case is reduced of roughly one third compared to that obtained by the site-specific ligand thiol coupling.



Supporting References

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