Unimolecular FRET Sensors: Simple Linker Designs and Properties

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***Abstract*—Protein activation and deactivation is central to a variety of biological mechanisms, including cellular signaling and transport. Unimolecular FRET probes are a class of fusion protein sensors, that allow biologists to visualize using an optical microscope whether specific proteins are activated due to the presence nearby of small drug-like signaling molecules or analytes. The focus of the present work is to explore through simulation the critical role of protein linker linking different parts of rather generic unimolecular FRET probes.**

I. Introduction

Measurement of biomarkers and ligands are increasingly used to study transport, signaling and communication in cells, and as diagnostics/prognostics of disease, or the presence of pathogens, allergens and pollutants in foods, and the environment. Accurate measurement in assays or cellular environments is important, and protein based biosensors can be used in this context. But due to the molecular complexity of such sensors,understanding the features that determine their performance is difficult both from the perspective of experiment, and detailed molecular dynamics. In the latter case this is due to the size of the system to be simulated and the associated time and spatial scales. To investigate such systems, at a qualitative level we use simple coarse grained models of proteins, and for critically important features requiring high accuracy, we employ advanced molecular dynamics, in particular rare-event methods.

Fluorescence (or Föster) resonance energy transfer (FRET) occurring between donor and acceptor fluorescent protein (FP) pairs can provide detailed spatio-temporal information about a wide range of biological processes. Typically, the FRET efficiency, $\mathcal{I}$ the average fraction of energy transfer events per donor excitation event - falls off quickly with distance between the FPs near the so called Föster radius, $R_0 \sim 5-7$ nm, thus offering a highly sensitive indicator of spatial and temporal change between the FP pair. Biosensors incorporating FP pairs can be designed to respond to variations in local concentrations of target analytes (small signaling molecules or biomarkers), that change the internal structure of the biosensor, bringing the FPs closer on average, which in turn can be observed optically through changes in the FRET efficiency.

Many unimolecular FRET based probes designed to monitor or report the local concentrations of analytes, comprise a donor FP attached to a ligand binding domain, a sensor or reporter domain attached to the acceptor FP, with these ligand binding and sensor domains connected by a linker (see Fig.1 for three examples). When the ligand binding domain is activated due to the proximity of a ligand or analyte (the so called ON state), an attractive interaction is turned on between the binding and sensor domains causing them to come together, bringing their donor and acceptor FPs closer. In the absence of the ligand/analyte (the OFF state), the domains should remain further apart. Such spatial changes can be measured by changes in the FRET efficiency between the FPs.

How well one can discriminate between the background or basal efficiency $\mathcal{I}_0$, and changes in the FRET efficiency due to changes in the analyte concentration close to the sensor is determined by the signal-to-noise ratio (also known as the dynamic range) $(\mathcal{I}-\mathcal{I}_0)/\mathcal{I}_0 = \Delta\mathcal{I}/\mathcal{I}_0$, and is of critical importance in sensor design. A related quantity is $\mathcal{J} = \frac{\sigma(\mathcal{I}-\mathcal{I}_\prime)}{\mu(\mathcal{I}-\mathcal{I}_0)}$ which is simply related to the so called Z' factor used to characterise the quality of a sensor. In particular, one can easily show (making reasonable assumptions) that the fractional error in the ligand/biomarker concentration predicted from calibrated FRET measurements is proportional to J. Here μ and σ denote the mean and the variance. This allows the effect of changes in the sensor design to be easily related to the accuracy at which concentrations of target ligands/biomarkers can be measured.

The choice of molecular linker used to connect the components B and B' of the biosensor depicted in the top panels of Fig. 1 can have a strong influence on its overall performance[1]. In this current work we first model the flexible linker system developed by Komatsua *et al.*[2] using a variable numbers of repeat units of the form $(\text{SAGG})_n$ to design a FRET biosensor for Kinases and GTPases. We then compare these results with idealized models of hinge type linkers built using α-helical proteins.

II. Results

The influence of different geometrical/structural properties of linkers on the FRET efficiency is explored here using simple statistical mechanics models and Monte Carlo simulations.

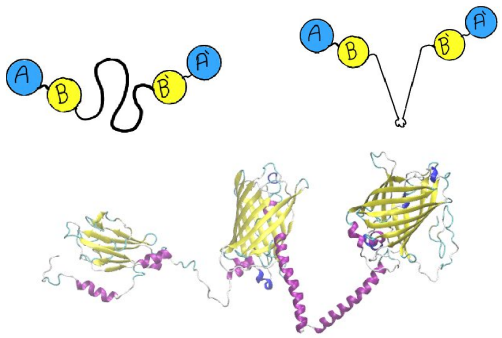

Fig. 1. Top-left is a schematic illustration of a unimolecular sensor where a flexible linker is used to connect protein modules B and B'. Top-right corresponds to the case where the flexible linker is replaced by a free hinge type protein. The bottom figure is an example of a PKA sensor where the yellow cylindrical-like proteins flanking the hinge are FP's; and the sensing units are the PKA substrate (far left) and corresponding consensus protein respectively (far right). When the PKA substrate is phosphorylated by PKA, it will bind to the consensus protein. Note frequently the order of A,A' and B,B' is interchanged.

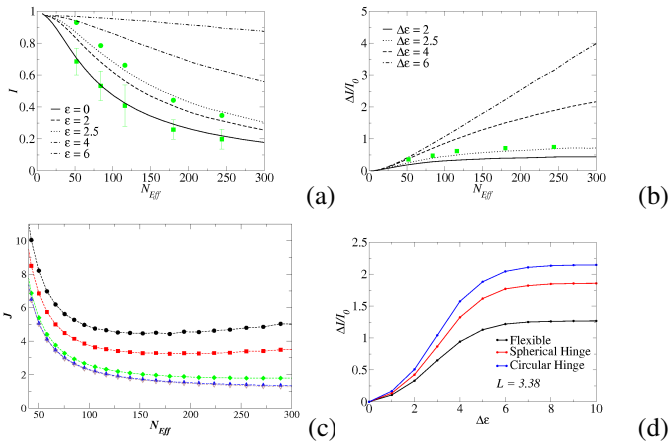

Fig. 2. (a) FRET intensity $\mathcal{I}$ of the flexible linker model as a function of number of linker residues. Results are presented for five different values of the binding energy ϵ, where $\epsilon = 0$ corresponds to the basal case. Data from experiments [2], in the OFF or basal state (filled green squares) and in the ON state (filled green circles) are superimposed on the theoretical predictions. (b) Corresponding signal-to-noise $\Delta\mathcal{I}/\mathcal{I}_0$ for the theoretical model overlaid with the experimental signal-to-noise ratio data (filled green squares). (c) $\mathcal{J}$ of the flexible linker model as a function of effective number of residues N_{Eff}. The lower the value of $\mathcal{J}$, the more accurate the sensor, where each curve corresponds to a value of ϵ, black bullet 2; red square 2.5; blue lozenge 4; green triangle 6; and down-triangle 8 (in units of k_BT). (d) $\Delta\mathcal{I}/\mathcal{I}_0$ is plotted for optimal hinge and flexible linker sensors respectively.

A. Comparison of Simulation & Experiment for the flexible linker

In Fig.2 we compare the results of our flexible linker model simulations with the experimental findings for both signal (a), and signal-to-noise ratio (b) obtained by Komatsua *et al.*[2] as a function of linker length. The comparison give consistent estimates for the ON state binding energy for this particular experimental system of $\epsilon = 2.5\ k_BT$. In panel (c) $\mathcal{J}$ is plotted as a function of effective number of residues N_{Eff}.

B. Comparison of performance of flexible and hinge linkers

To compare the performance of sensors when the flexible linker between B and B' is replaced by a free hinge, we demanded that the arms of each hinge consist of at most 30 residues each so as to ensure their structural stability, and that the flexible linker correspond to the optimal linker of Komatsua, which was 116 residues in length. Fig 2(d) shows that under such assumptions, the signal to noise ratio's where free hinge linkers are used instead of flexible linkers are significantly higher. In fact, the hinge proteins used by Boersma et al behave as free spherical hinges (the detailed free energy simulation results are not displaced here due to space limitations).

III. Methods

To analyze experimental FRET microscopy results, we have built simplified models of unimolecular FRET probes, represented by two "macro-particles" joined by an idealized linker. The macro-particles are connected to either end of a peptide linker, which may be flexible, or "hinge-like" modeling secondary structures such as a pair of flexibly connected alpha helices [3].

The spherical macro-particles interact through a pair potential of the form

$$V(R) = V_s(R) + V_\ell(R) \tag{1}$$

where the first term denotes binding between the macro-particles due to the presence of the target ligand/analyte, and the second term is an interaction specific to each linker type. The resonance energy transfer rate ($\mathcal{I}$) is computed by averaging

$$I(R) = \frac{1}{1 + (R/R_0)^6} \tag{2}$$

for each system using Monte Carlo simulation for the corresponding potential, where the Föster radius $R_0 \sim 5 - 7$ nm giving the distance at which the energy transfer efficiency is 50% and R is the distance between the spherical macro-particles.

IV. Outlook

It is possible to extend this work to develop protein based molecular switches which enhance precision by more than an order of magnitude, to be reported in print elsewhere.

Acknowledgment

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