New and Notable

Molecular-Scale GPS: Positioning a Biosensor Peptide on RyR

Suren A. Tatulian[1,*]
[1]Department of Physics, University of Central Florida, Orlando, Florida

You might have wondered how the Global Positioning System (GPS) manages to pinpoint whether your car is at the parking lot or on a nearby road just 10 feet away. The official site of GPS.gov reports that high-quality receivers provide >3 meter horizontal accuracy, i.e., the dimension of a medium-sized car; a remarkable spatial resolution! Some might have wondered if it would be possible to use the principle of GPS to determine the location of a molecule, e.g., an effector peptide, relative to a target protein, e.g., an ion channel; a challenging task that will require improvement of the resolution by a factor of 10^{10}. Efforts by several research groups, including a study by Svensson et al. (1) published in this issue of the *Biophysical Journal*, have resulted in a multistep method reminiscent of GPS to characterize protein structure or to position one molecule relative to another. Fluorescence resonance energy transfer (FRET) is used to obtain multiple distance constraints, which are then analyzed in a trilateration (or triangulation) procedure to locate the components of a molecular complex relative to each other. The purpose of this New & Notable is to hit the high spots of these studies and to briefly discuss the advantages and limitations of this new biophysical toolkit for macromolecular characterization.

Muschielok et al. (2) utilized this approach, which they termed a Nano-Positioning System, to locate the nascent RNA in yeast RNA polymerase II elongation complexes and the effect of the transcription factor IIB. They measured FRET between an acceptor-labeled site (antenna dye molecule) and several donor-labeled sites (satellite dye molecules), followed by triangulation of FRET-derived distances and positioning of the acceptor site within the macromolecular complex.

FRET-based distance determination has been used to gain structural information on type-1 and type-2 ryanodine receptors (RyR1 and RyR2), which are Ca^{2+} release channels in the sarcoplasmic reticulum membranes of skeletal and cardiac muscle cells, respectively (3–5). Dysfunction of RyR has been linked to serious pathologies such as ventricular tachycardia and heart failure (6); hence, a better understanding of its structure and function is of great biomedical importance. RyR1 and RyR2 share 66% sequence identity and both are homotetramers of a total molecular mass of ~2.3 MDa, making them the largest ion channels known. No atomic resolution structures of the full-length channel molecules have been reported, but the cryo-electron microscopy (cryo-EM) reconstructions of RyR2 (7) and RyR1 in the open and closed states (8) have been determined at ~30 and ~10 Å resolutions, respectively. The tetramer is an immense mushroomlike molecule ~160 Å high (Fig. 1 *A*) with a head that is viewed from the cytoplasm as a 270 × 270 Å square (6,8). Domain movements upon channel gating have been suggested (6,8). However, much of the contemporary knowledge on the domain structure of RyR, the secondary and tertiary structural features of the domains, the mode of binding of accessory proteins, and conformational changes that underlie channel gating, is gained mostly from computational approaches that need experimental testing.

If channel gating involves changes in interdomain distances, then measurements of such distances in the open and closed states would help us to understand the structural mechanism of the channel. In an attempt to address this issue, Raina et al. (3) developed an approach where green fluorescent protein (GFP) is engineered into a selected site of RyR1 while a 10-residue histidine tag (His-tag) is inserted into another site, followed by binding of a His-tag specific dye Cy3NTA that pairs with GFP as an energy acceptor. This method was used to estimate the FRET-based distances between GFP placed at three different sites of RyR1 and Cy3NTA at five sites, and the positions of GFP were determined by triangulation relative to a 559-residue fragment of RyR1 with known crystal structure. The structural information gained from these experiments was limited, or more qualitative than quantitative, for a range of reasons. Most notably, the large sizes of both the donor and the acceptor probably caused structural perturbations in the RyR1 protein and also prevented an accurate positioning because of uncertainties in conversion of FRET efficiencies to distances. It was recognized that other, less invasive labeling strategies were required (3).

An alternative FRET-based method has been developed that relies on RyR modulator proteins labeled with donor and acceptor probes; binding of these molecules to RyR allows FRET measurements without affecting RyR structure (1,4,5). Two major RyR modulators have been studied, i.e., FK506 binding protein (FKBP), which promotes the closed channel conformation, and calmodulin (CaM), which exerts distinct Ca^{2+}-dependent functional effects on the RyR1 and RyR2 channels. Both tightly bind to RyR1 and RyR2 and are considered endogenous regulators, or even constitutive RyR subunits, that keep cytosolic Ca^{2+} under control. Ca^{2+}-free and Ca^{2+}-bound CaM's had been shown

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*Correspondence: statulia@ucf.edu

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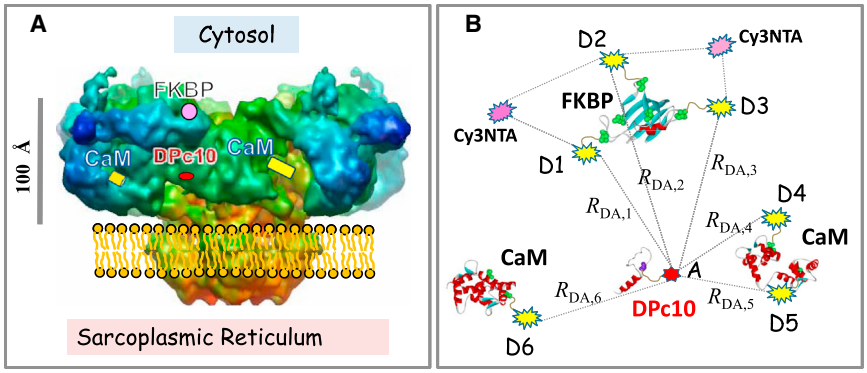

FIGURE 1 (*A*) Three-dimensional cryoEM reconstruction of RyR1-FKBP12 complex in the closed conformation (EMDB:1606, Samsó et al. (8)). Side view colored by cylindrical radius, surface level: 1.95, image by the software UCSF CHIMERA (University of California at San Francisco, San Francisco, CA; https://www.cgl.ucsf.edu/chimera/). The sarcoplasmic reticulum membrane and bound FKBP, CaM, and DPc10 molecules are shown schematically. (*B*) Cartoon for a system of multiple FRET pairs to position the DPc10 peptide. (*Yellow polygrams*) FRET donors attached to FKBP or CaM are labeled D1–D6; (*magenta polygrams*) His-tag-attached Cy3NTA FRET acceptors form FKBP; (*red heptagram*) DPc10-attached FRET acceptor from FKBP or CaM is labeled *A*. Amino-acid residues in FKBP and CaM and the N-terminal residue of DPc10 shown in ball-and-stick format (*green* and *purple*, respectively) are those substituted with cysteine for dye attachment. The structures of Ca^{2+}-loaded CaM complexed with a RyR1-derived peptide and FKBP are based on PDB:2BCX and PDB:1D6O, respectively, and DPc10 is shown in a predicted conformation. $R_{DA,i}$ are the distances between the donors and acceptors. The fluorescent labeling scheme is simplified to show the principle of the method. See text for more detail.

by cryo-EM to bind to distinct sites of RyR1 (9). The cryo-EM reconstruction of RyR1 with bound FKBP is also available, and the crystal structure of FKBP had been docked into the respective electron density (8). Yet more detailed descriptions of the binding modes and interfaces of these accessory proteins are required to understand the mechanism of channel regulation. Cornea et al. (4) used natural RyR1-containing sarcoplasmic reticulum membranes and allowed FKBP and CaM, labeled with Alexa Fluor (Invitrogen, Carlsbad, CA) FRET donors and acceptors, to bind to RyR1, followed by distance measurements between one site of FKBP and three sites of CaM. The measured distances showed little Ca^{2+}-dependence, which contrasted the predicted large-scale Ca^{2+}-induced conformational changes in CaM. The data nevertheless allowed docking of the crystal structure of Ca^{2+}-CaM in complex with a RyR1-derived peptide into the cryo-EM map of RyR1, thus positioning CaM on RyR1 with a defined orientation.

The next step was to position FKBP on RyR1 and RyR2. Five single-cysteine FKBP constructs were created and labeled each with Alexa Fluor FRET donors, while a CaM mutant T34C was labeled with an Alexa Fluor acceptor (5). Four out of five Alexa Fluor-labeled FKBP molecules tightly bound to RyR1 and RyR2 and produced robust FRET effects, allowing determination of four FKBP-CaM distances ranging 61–77 Å. This work showed that FKBP isoforms bind to equivalent sites of RyR1 and RyR2 at similar orientations.

Further efforts were directed to test a proposed domain zipping/unzipping mechanism of RyR function according to which the closed channel state is stabilized by interaction (zipping) between the N-terminal (residues 1–600) and central (residues 2000–2500) domains of RyR. Consistent with this mechanism, a peptide corresponding to the central domain sequence 2460–2495, named DPc10, was able to potentiate Ca^{2+} leakage in ventricular myocytes and arrhythmogenic mutations such as R2474S reversed this effect (10).

The work by Svensson et al. (1) is an in-depth analysis of binding of DPc10 to RyR2 in ventricular myocytes. The cells were detergent-permeabilized and loaded with FKBP labeled at five distinct sites with Alexa Fluor FRET donors, and DPc10 labeled with a FRET acceptor at its N-terminus. In separate experiments, RyR1 constructs that contain His-tag inserts at four distinct sites at the N-terminal domain were expressed in HEK cells and loaded with donor-labeled FKBP and Cy3NTA that binds to the His-tags and serves as FRET acceptor at defined locations. A simulated-annealing method was used to refine the FKBP-attached donor positions, which were validated using FRET measurements between all five FKBP donors and all four Cy3NTA acceptors in HEK cells. FRET efficiencies from five sites of FKBP to DPc10 were then measured in cardiomyocytes, and trilateration was applied to position the acceptor attached to the DPc10 peptide.

Finally, the positioning of DPc10 was refined by additional constraints imposed by previously measured distances between RyR2-bound CaM N- and C-lobes and DPc10 (Fig. 1). The simulated-annealing procedure, which restricts the number of possible dye positions by taking into account the proximal protein molecules and the torsion/energy parameters of the linker, minimized the errors stemming from the relatively long and flexible linkers. Thus, multiple checkpoints have been set to bring all existing data into agreement. Yet the trilateration procedure was not an easy one. Combination of all five FKBP-to-DPc10 distances did not yield a reasonable location for DPc10, and only one out of five combinations of four distances resulted in a meaningful position of the DPc10 peptide at the RyR domain 3, the "handle" that connects the FKBP and CaM sites, and importantly, ~35 Å from the N-terminal domain. This latter finding certainly challenges the domain zipping/unzipping hypothesis and calls for more studies.

The features that distinguish this work are a comprehensive, multiscale analysis and critical assessments of the accuracies of all steps that led to the final molecular positioning. The RyR channel complex has been studied in an intact form in its natural membrane environment in myocytes, which is advantageous compared to situations where the membrane proteins are being recombinantly overexpressed, purified, refolded, and reconstituted in artificial lipid bilayers. The FRET probes were targeted using FKBP and CaM, which are near and dear to RyR and hence form a natural complex with it without structural perturbations. FRET is superior in its capability of evaluating much larger intra- or intermolecular distances than NMR. Yet there is room for methodological improvements. Long and flexible linkers introduce serious problems regarding the precise locations of the fluorophores. Using shorter linkers and determining the transition dipole orientations by fluorescence anisotropy measurements would be a better strategy. Also, measuring distances between multiple donor sites and a single acceptor site does not determine the mode of the peptide binding; the peptide retains a wobbling freedom within a hemisphere with a radius of its own size.

In closing, FRET-based methods hold the promise of macromolecular structure characterization, especially when size or flexibility issues keep them out of reach of more conventional techniques such as NMR or x-ray crystallography. Alternative labeling strategies may be considered in future studies. For example, instead of attaching relatively bulky probes to cysteines engineered into defined sites of a protein using long linkers, insertion of unnatural amino acids with altered fluorescence properties as FRET donors and acceptors may be used (11). Preparation of just two tRNAs with nonsense or frameshift suppressor anticodons and charged with unnatural amino acids that make a FRET pair, plus a cDNA that incorporates respective triplet or quadruplet codons at desired sites, will do the job. The procedures that are less invasive and provide maximum information at minimum effort and investment may be given highest priority.

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