Determining Structural Mechanisms of G Protein-Coupled Receptor Allostery

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Introduction: G protein-coupled receptors (GPCRs) transduce the information of soluble ligand binding into intracellular signaling responses critical for cell communication. With crystal structures of GPCRs solved only recently[1], our understanding of the structural basis of signal transduction for these receptors is far from complete. Recent spectroscopic studies provide evidence for conformational states not observed in crystal structures, as well as substantial conformational heterogeneity upon binding different ligands[2]. Static crystal structures cannot describe the dynamic characteristics of GPCR activation; thus, our current understanding of the dynamic structure-function relationships between extracellular ligand binding pockets and the intracellular G protein binding site is limited, especially in regard to allosteric ligands, which bind at a site other than the classical orthosteric (endogenous) ligand binding site.

Allosteric ligands can increase or decrease GPCR signaling strength by altering orthosteric ligand affinity and/or ligand efficacy, or by direct modulation of signaling[3] (Fig 1a). Intriguingly, the behaviors of an allosteric ligand (for example, alcuronium) can vary depending on which orthosteric ligand is bound. I propose determining the structural basis for these behaviors since it is unknown how positive versus negative allosteric modulators stabilize specific receptor conformations. I will also address the question of whether structural changes are concerted (indicating coupling) between the distinct allosteric, orthosteric, and G protein binding domains.

Hypothesis: Allosteric modulators alter the ensemble of GPCR conformations; allosteric ligands that primarily alter orthosteric ligand affinity will stabilize (or destabilize) the same ensemble of active states as observed with orthosteric ligand alone. Allosteric ligands that primarily alter efficacy will preferentially stabilize just one of the multiple possible active conformations. I predict allosteric activation increases the rigidity between the extra- and intra-cellular domains.

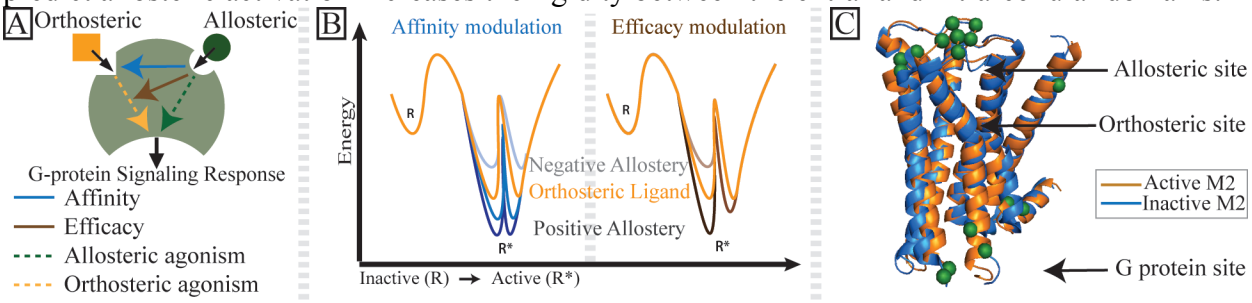

Figure 1: a) modes of GPCR activation; b) reaction coordinate measured by EPR immobile/mobile spectra (cwEPR, Aim 1) and TM4-TM6 distance populations (DEER, Aim 2); c) M2 receptor with introduced cysteine label sites.

Methods and model system: The dynamic character of GPCRs and the low affinity of most allosteric modulators limit the utility of GPCR crystallography in the study of allosteric activation. Conversely, the techniques of continuous wave electron paramagnetic resonance spectroscopy (cwEPR) and pulse field EPR (double electron-electron resonance or DEER spectroscopy) can capture the dynamics of conformational transitions, allowing the description of GPCR conformational changes and identification of intermediate states that are unlikely to be crystallizable[4]. cwEPR uses free radical spin-labeling of cysteine side chains to measure differences in the relative mobility of the labeled residue. DEER spectroscopy requires labeling of two cysteines to measure the distribution of distances between two domains. Facilitating EPR probe design, the Kobilka lab has recently solved crystal structures of inactive[5] and active states of the M2 muscarinic receptor both bound and unbound to a positive allosteric modulator (Kruse et al., *Nature* in press), identifying the topologically distinct allosteric binding site. I used these crystal structures to design M2 cysteine mutant proteins (Fig 1c). Additionally, I have developed

a HEK cell expression system to efficiently produce mutant proteins, demonstrated expression of functional mutant M2, and prepared a minimal cysteine construct to serve as a control for site-specific spin labeling studies. After expression of each cysteine mutant, I will verify the functionality by radioligand binding and activation assays[2] to isolate ligand-specific effects. This cysteine mutant system will allow EPR characterization of states stabilized by allosteric ligands.

Specific Aim 1: *Determine whether novel conformations are stabilized by allosteric ligands that alter orthosteric efficacy.* Allosteric ligands may function by changing the equilibrium of active states or by stabilizing distinct conformations (Fig 1b). To distinguish between the two models, I will spin label extracellular and intracellular residues that are revealed by crystal structures to undergo movement upon activation. With cwEPR, which can sensitively detect small distance changes (<25 Å), I will monitor the movements of transmembrane (TM) helices relative to each other in the presence of different allosteric modulators: those that affect signaling via changes in orthosteric ligand efficacy (including rapacuronium(-), alcuronium (+)) or affinity (gallamine (-), alcuronium (+)). I will correlate local mobility changes in the helices with the degree (+/-) and type (efficacy/affinity) of ligand by comparing EPR spectral shifts with measured signaling pathway outputs of each allosteric compound and with the available crystal structures of active and inactive M2. If distinct or novel spectra are not observed for allosteric/orthosteric ligand combinations, this will indicate a model whereby allosteric ligands alter equilibrium populations (affinity model) rather than preferentially stabilize a subset of conformations (efficacy model).

Specific Aim 2: *Determine whether the allosteric site communicates with the orthosteric and ligand-binding sites through rigid-body movement.* To quantify the larger (18-60 Å) structural changes upon allosteric activation, I will use DEER spectroscopy, a technique that has been previously used for rhodopsin to demonstrate rigid-body movement of the seven TM helices[6]. In contrast to rhodopsin, recent evidence from studies of the β2 receptor suggests ligand-activated GPCRs have weaker structural coupling between the orthosteric and the G protein binding sites[2]. To determine the coupling mechanism between sites, I will assay the population distribution of distances between TM4 and TM6, which have been shown to move significantly upon activation. A rigid-body activation model, with structural coupling between domains, would be indicated by concerted distance population distribution changes at allosteric and G protein sites. A lack of concerted change would suggest a looser structural coupling unaffected by allosteric ligands. I predict that any concerted distance changes will depend on the degree and type of allosteric and orthosteric ligand combinations, which would support a mechanistic model for allosteric activation through altering helix rigidity. DEER analysis of an apo receptor (allosteric ligand only) would then determine whether a rigidity mechanism exists for direct allosteric agonists. An important control for DEER studies will be the use of a characterized antibody fragment to lock the receptor in a single conformation, providing a baseline distance distribution.

Summary and Broader Impacts: I will use the GPCR family as a model system to understand the unique protein property of allostery. My findings will allow development of new cell-based technologies where the activity and signaling pathways for GPCRs and other allosteric proteins may be exquisitely fine-tuned by rational design of allosteric ligands. The experiments I have proposed will define the structural contribution of allosteric modulators to GPCR signaling responses and will capture novel ligand-induced conformations and activation dynamics that cannot be observed by crystallography. I will share my passion and expertise for this topic by mentoring an undergraduate student; the parallel mutant generating steps are ideal for teaching.

References: **1.** Venkatakrishnan, A. J. *et al. Nature* 494, (2013); **2.** Nygaard, R. and Kobilka, B. *Cell* 152 (2013); **3.** Conn, J. *et al. Nat. Rev. Drug Discov.* 8 (2009); **4.** Hubbell, W. *et al. Nat. Struct. Mol. Biol.* 7 (2000); **5.** Haga, K. *et al. Nature* 482 (2012); **6.** Altenbach, C. *et al. PNAS.* 105 (2008).

Rachel Matt, Kobilka Lab, Stanford University

My passion for discovering and sharing scientific information was cultivated underground, during four summers spent guiding tours through a dark and damp limestone cave in Minnesota. As a young and shy high school student, this summer job taught me how to convey complicated scientific concepts to a diverse audience in an educational and yet interesting manner. During my hour-long walks through the caverns, I engaged hundreds of tourists in learning the rules of the underground world: sedimentation and bedrock geology, fossilization, and the chemistry of stalactite and stalagmite formation. The summer job led me on a path to study chemistry and to educate younger students as a chemistry tutor and undergraduate teaching assistant. In the classroom, I gave presentations about chemical and physical concepts and then led students in hands-on experiments to apply what they learned. Teaching my fellow undergraduates built on the communication skills I learned during the cave tours, reminding me how important it is to complement analogies with visual demonstrations to make difficult concepts more approachable.

These teaching experiences both motivated me towards a future career in academia and prepared me to fulfill my goals in broader scientific outreach as a graduate student. I am excited to continue the hands-on demonstrations of scientific concepts through the Stanford Splash outreach program, an annual teaching weekend which brings together middle and high school students from all over the country, with funding for students from financially disadvantaged backgrounds. During the Splash program, I will teach courses about crystal diffraction and the behavior of chemicals and proteins in magnetic fields, applying the skills I learned in my previous teaching experience for giving engaging, interactive demonstrations. Participating in the Splash program will give me practice in making my research topics accessible to a lay audience, as well as engage diverse students in a hands-on science research project. Additionally, I will share my career path and current research this spring at the Bridges STEM Speaker Series, a local community college program that provides support for underrepresented minorities to pursue degrees in biomedical sciences. The goal of this program is to connect these college participants with current graduate students in the biosciences. I am excited to share my unique experiences during this seminar series, including how my career goals as a scientist were shaped at a small liberal arts institution and as a pharmaceutical industry employee.

As an undergraduate, I spent three years in a volunteer research position in the Priestley Lab at the University of Montana where I studied bacterial natural products. I investigated the polyketide synthase (PKS) biosynthetic pathway of *Saccharopolyspora spinosa;* I attempted to modify the natural product structure through both biological and chemical manipulations to generate a compound library that could be screened for antibiotic activity. We reasoned that a library based on this scaffold could have novel antibiotic properties since *Sacc. spinosa* is closely related to *Sacc. erythraea,* which produces the antibiotic erythromycin through a PKS pathway. To explore the structural diversity of a library based on this natural product scaffold, I independently generated the compound library virtually by encoding the structure in a computational string. I used a principal components analysis to reveal a subset compounds in the potential library that encapsulated the diversity of the entire set. This allowed us to select future target molecules for chemical modification of the scaffold before engaging in costly synthesis and screening. I also honed skills in organic chemistry and molecular biology by performing the genetic manipulation of *Sacc. Spinosa* for scaffold generation. I presented my results in a written final honors research report and in a poster at the UM Conference on Undergraduate Research.

I was able to follow my passion for chemistry applied to biological systems upon graduation, when I worked at GlaxoSmithKline for three years in a vaccine adjuvant

manufacturing and research group. Here, I saw one example of how basic scientific research could be applied to benefit society. I worked closely with team members on the drug manufacturing line and continued teaching my fellow scientists by becoming a qualified trainer for the manufacturing process. This team-based role was rewarding as well as challenging, as I had to communicate manufacturing concepts and techniques to coworkers who were often much older and more experienced than myself. Later, working in a formulations and drug delivery research group, my supervisors expected me to define my own role in the project and to set my own deadlines. My responsibilities were to develop formulations for aqueous delivery of synthetic molecules and to quantify the stability of these molecules through analytical chemistry techniques. I learned quickly that working in this environment required me to be able to independently troubleshoot problems with equipment and techniques. In one instance, several attempts had previously been made at improving the water-solubility of a class of vaccine adjuvant molecules. Even though other research group members felt that we would not be able to improve the intrinsic solubility of these molecules, I saw potential based on my research of the formulations literature. I designed a high throughput screening method that tested a variety of cosolvent formulations, which resulted in a modest solubility improvement. Encouraged, I applied the screening technique to novel salt forms of the synthetic molecules, one of which improved the solubility of our lead compound over 40-fold, allowing the compound to be tested in vivo. Through this process, I learned how to research the literature and synthesize my findings into an effective experimental design. Yet my most important lesson was in perseverance; I learned the rewards of following through on an idea, even when past attempts are unsuccessful.

Throughout my time as a member of the formulations research group, I collaborated daily with both medicinal chemists and immunologists. I learned to speak the languages of both disciplines, and I found myself becoming an ambassador between the two groups, able to relay project information back and forth since I was working with them daily. I also endeavored to expand the outreach of my formulations group by organizing a chromatography forum that brought together international GSK scientists who worked with analytical chemistry techniques for monthly meetings where we could share our expertise and optimize methods. I continued to expand my scientific communication skills by presenting my formulations work to collaborators both locally and internationally during monthly oral presentations. Working with such a large and diverse group of researchers made me excited to continue such collaborations to bridge chemistry and biology during my graduate career.

One final lesson I took away from my work experience was an understanding of the importance of strong basic science in an applied research program; I found that where the basic physical and biological understanding was weak in our vaccine adjuvant program, there were many setbacks to the research experiments and timeline. Thus, my work experience both motivated me to continue my own education in the basic sciences and cultivated my interest in the scientific areas of cell signaling and receptor biology. The Kobilka Lab at Stanford University was the ideal choice for my graduate thesis work, where I will apply biophysical techniques to understand the structural basis of protein allostery, with G protein-coupled receptor (GPCR) signaling as a model system. My proposed work will reveal ways to design allosteric ligands for fine control over cell signaling systems. By working in the field of GPCR biochemistry, I will develop expertise in many technical disciplines and will have the opportunity to develop new methods to understand protein structure-function relationships in even finer detail. My work to date in the Kobilka Lab has already set me on the path towards accomplishing these goals: during my rotation in the lab, I completed two projects. Firstly, I

expressed fusion proteins of a GPCR to generate antibody fragments against the extracellular surface of an active receptor. We predicted that the fusion protein would stabilize the activated conformation of the receptor for immunization, which I confirmed with a fluorescence biochemical assay. The antibody fragment we will generate will be a useful tool for future structural studies. In my second project, I fused a crystallization aid protein, a novel derivative of T4-lysozyme, to an intracellular loop of the M3 muscarinic receptor, then successfully crystallized and solved the structure. The new T4-lysozyme generated by this project will be broadly useful in crystallography projects where a variety of crystal packing lattices are needed, and I am currently preparing this work for publication.

The NSF GRF will support my continued research in Brian Kobilka's lab, where I will learn both the important questions to be answered in the field of GPCR structural biology, as well as the skills to carry out my proposed research. Specifically, my research will involve biophysical assays, including electron paramagnetic resonance (EPR) spectroscopy. In order to perform these experiments, I will need to use biochemical techniques for membrane protein expression, purification and characterization, many of which I have already gained experience with during my first few months in the lab. Our collaboration with Dr. Wayne Hubbell at UCLA will provide the necessary resources for pulse-field EPR experiments. I hope to continue working in this or a related biophysical field in my future career, and I see my thesis work as another opportunity for outreach by involving undergraduate students, perhaps though my connections formed through the Bridges STEM Speaker Series. My proposed project requires the generation and analysis of many mutant proteins; these are ideal experiments for teaching undergraduates, as they can be carried out in a parallel pedagogic fashion.

In addition to the technical skills I will acquire as an NSF fellow, I hope to develop my professional skills as a scientist. My goals in this area are to improve my project planning and management skills along with my teaching and mentoring abilities. To this end, I have sought mentorship through campus-based mentoring programs supported by the Association for Women in Science (AWIS), and Women in Science in Engineering (WISE) groups. In my second year, I have begun to give back to these mentorship programs by serving as a peer mentor and officer in the Stanford Biosciences Student Association. I have also begun to develop skills in project management by participating in the Stanford SPARK program. SPARK brings together academicians, pharmaceutical industry workers and venture capitalists to provide funding and education, with the goal of quickly moving research projects from the university into clinical trials. During my coursework with SPARK, I have worked with a small team of fellow students to develop an idea for treating a pediatric disease; we spent 20 weeks researching the field, planning the required basic research, and developing the appropriate clinical trial for our proposed drug, which culminated in a successful pitch to the SPARK advisory board. The process was an invaluable experience in thoroughly planning a complete research project by thinking deeply up front about the ultimate research goals.

An NSF GRF will allow me to take advantage of these opportunities and more, where I will continue to learn project management, public presentation, and networking skills. The extraordinary opportunities available to me as a graduate student at Stanford will allow me to make contributions in three important areas: scientifically, by expanding methods and knowledge of the fundamental property of protein allostery; educationally, through outreach teaching and mentoring programs; and personally, by forming me into a more engaged community member and more competent scientist, capable of achieving my career goals.

Intellectual Merit Criterion

Overall Assessment of Intellectual Merit

Excellent

Explanation to Applicant

GPA 3.98/4 at U Montana Grad at Stanford with 4.06/4 GPA Lots of research experience including 3 years after college before graduate school at GlaxoSmithKline Graduate adviser is 2013 Nobel Laureate Good research proposal

Broader Impacts Criterion

Overall Assessment of Broader Impacts

Very Good

Explanation to Applicant

Peer mentor for graduate students chemistry tutor and teaching assistant in college Involvement in science outreach to K-12 through Stanford SPLASH Limited evidence of past outreach experience

Summary Comments

Strong student with lots of research experience and good plans for future outreach.

Intellectual Merit Criterion

Overall Assessment of Intellectual Merit

Excellent

Explanation to Applicant

This is a very well written proposal with clear aims that describes the experimentation that will be performed. Her credentials are impeccable: superior academics that have been routinely recognized through many awards; undergraduate research experience that resulted in numerous presentations; industry research experience; and second authorship on a submitted manuscript from her graduate work after a short time in her new laboratory.

Broader Impacts Criterion

Overall Assessment of Broader Impacts

Excellent

Explanation to Applicant

The applicant has a history teaching about science to a broad audience from her high school/college job as a natural history guide. Now, she will continue to develop that sense of outreach by working with a local science education program that brings together teachers and students from diverse backgrounds to give them intensive workshops on scientific concepts. Additionally, she will hone her leadership skills through involvement with organizations that reach out to women in science. Importantly, her research program is timely and important, broadening the impact of her proposal beyond social outreach to having a real potential to contribute fundamental understanding of protein dynamics.

Summary Comments

This is an excellent application by a very well qualified applicant.

Intellectual Merit Criterion

Overall Assessment of Intellectual Merit

Excellent

Explanation to Applicant

The applicant proposes to study G proteincoupled receptors (GPCRs) to study the fundamental property of allostery. To determine structural mechanism of GPCRs, Rachel proposes to use state-of-the-art biochemical and biophysical techniques, including Electron paramagnetic resonance (EPR) spectroscopy. She intends to use double electron-electron resonance (DEER) spectroscopy to study whether the allosteric site communicates with the orthosteric and ligand binding site-through rigid-body movement.
Rachel plans to continue her work with Dr. Kobilka who is a leading expert in the field.

Broader Impacts Criterion

Overall Assessment of Broader Impacts

Excellent

Explanation to Applicant

Project will provide deeper insight into the mechanism of activation of G protein coupled receptors. The study will provide direction for rational design of novel allosteric ligands. Currently there is no understanding in this regard.
The applicant actively participates in AWIS and WISE groups and also plans to disseminate results of her research to the broader community. She will also continue to engage and share her expertise with underrepresented students through Bridges STEM speaker Series.

Summary Comments

There is a considerable interest in GPCR allosteric modulators. However, the field is facing several challenges- we do not yet understand the molecular mechanism precisely. The proposed study will answer important questions in the field of GPCR biology.
The work Rachel proposes to do, to accomplish the goals of the study are clearly set out and seem to be quite realizable. In addition, the applicant has a very strong research background supported with prestigious awards and honors.