COVER SHEET

TITLE: Measurement of The ADCK3 Transmembrane Helix Self-Association In *Escherichia Coli* Via the TOXCAT Assay

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YEAR: 2014

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The interaction of α-helices in the transmembrane region of certain proteins is important for the function of these proteins. The goal of our project is to study the self-association propensity of the transmembrane region of AarF Domain Containing Kinase 3 (ADCK3) and map its interaction interface. ADCK3 is a putative human mitochondrial kinase predicted to form a functional dimer. We measured the strength of the transmembrane helix-helix dimerization of ADCK3 via TOXCAT. Our results show that the transmembrane domain of ADCK3 forms a strong homo-oligomer due to the formation of the Cα-H hydrogen bonds between two amino acid residues. Site-directed mutagenesis is then carried out to map the interaction interface of ADCK3 to determine the amino acids that are important for self-association. From this experiment, we have also shown that the interaction interface of ADCK3 contains a Gly-zipper motif, a signature motif responsible for association in transmembrane regions of proteins.

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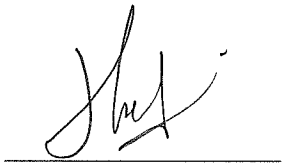

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ACKNOWLEDGEMENT

Foremost, I want to express my gratitude to Professor Senes for the opportunity to work in his laboratory. He has provided me with support, motivation and encouragement throughout the one-year experience I have had in his laboratory. I would like to extend my utmost appreciation to my mentor, Ambalika Khadria for her guidance and I am grateful for the learning opportunity. She has offered me with assistance in numerous ways and I am thankful for her dedication in helping me to complete my research project. Ambalika Khadria's teaching has been important in my academic development and it is vital for my future career development in the research industry. I have learnt essential laboratory skills from her and I believe the skills that I have learnt in Senes Lab are crucial in preparing me to be a successful scientist in the future. The completion of my thesis would not have been possible without the help and guidance from Professor Senes and Ambalika Khadria. I thank the University of Wisconsin-Madison, a world-class learning institution for the wonderful years and valuable experiences.

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ABSTRACT

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INTRODUCTION

Integral membrane proteins (IMPs) are attached to the lipid bilayer of membrane proteins and traverse the lipid bilayer. In general, IMPs facilitate the diffusion of molecules across biological membranes and it acts as channels to allow the transport of certain substances across the cell. Topologically, IMPs are divided into two categories, single-pass and multipass transmembrane (TM) proteins. Single-pass TM proteins pass through the lipid bilayer only once whereas multipass TM proteins pass through the lipid bilayer multiple times. Cytochrome P450 is an example of a single-pass transmbrane protein and a multipass TM protein includes glucose transporter 1 (GLUT1) (Lodish et al., 2000). In addition, there are two distinct classes of TM proteins, α-helical and β-barrel. The interaction of α-helices in membrane proteins assists in the folding and stabilization of the protein. The homo-oligomerization of TM helices or the association with other TM helices to form helical bundles are important for the function of the protein (Senes et al. 2004). The folding of α- helical membrane involves a two-step mechanism as proposed by Popot and Engelman (1990). Helices are formed and inserted into the membrane in the first step. In the second step, the helices associate and form the final folded structure.

Studies have been carried out to study the features that stabilize the interactions between TM domains of membrane proteins. The association can be driven by van der Waals interactions, hydrogen bonding and the hydrophobic interactions of the TM. An early study on glycophorin A (GpA) TM dimer done by Russ and Engelman (2000) shows the role of the GxxxG motif in mediating the interaction of TM helices. The GxxxG motif is found in the TM that has two glycine residues in between any three amino acid residues. Some of the biological examples include ErbB receptor, F_0F_1-ATP synthase, and integrins. The GxxxG-like motif, in which one or both glycine residues are substituted by another small residue (Ala, Ser), is important for the TM

helix association. It has also been shown that the homo oligomerization of the TM region also favors the formation of Cα-H···O hydrogen bond (Meuller & Subramaniam, 2014).

The goal of our project is to study the self-association of the transmembrane region of AarF Domain Containing Kinase 3 (ADCK3) and map its interaction interface. The TM region of ADCK3 is a putative human mitochondrial kinase predicted to form a functional dimer (Xie et al., 2011). ADCK3 is a human mitochondrial protein homologous to the yeast coenzymeQ8 (CoQ8) and the bacterial ubiquinone biosynthesis (UbiB) proteins. It is involved in the biosynthesis of CoQ (ubiquinone) in humans (Lagier-Tourenne et al., 1998). Research carried out by Lagier-Tourenne et al. has shown that CoQ deficiency is associated with recessive ataxia.

The strength of the TM helix-helix dimerization of ADCK3 is measured via the TOXCAT assay that uses a construct composed of the DNA binding domain ToxR, TM region of interest, and maltose binding protein (MBP) that functions as periplasmic anchor as shown in Figure 1 (Russ & Engelman, 1999). The periplasmic anchor of the MBP is essential for the correct topology of insertion of the TM peptide. The ToxR domains in cytoplasm are fused to the TM region of interest at the N-terminus. Transmembrane helix-helix association results in the dimerization of ToxR that

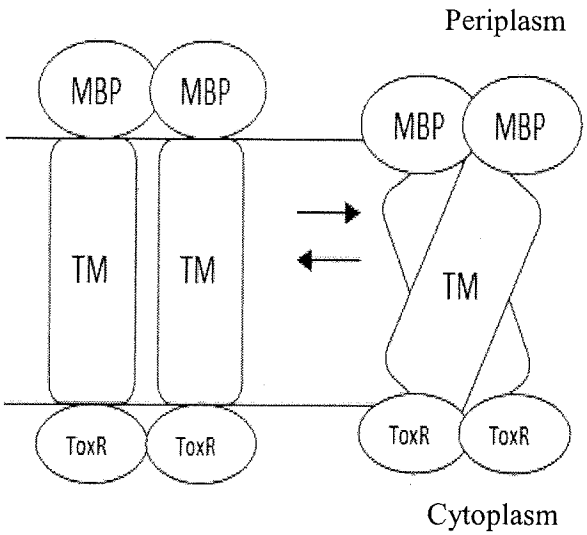


Figure 1. The TOXCAT construct. It consists of MBP, TM region of interest, and ToxR. The dimerization of the transmembrane peptide in *E.coli* shown increases the stability of the protein.

subsequently activates a promoter for a reporter protein, chloramphenicol acetyltransferase (CAT). An enzyme assay is then used to quantify CAT by measuring the level of its activity via UV/Vis spectrometer. The measured CAT activity is correlated to the strength of dimerization of the TM region. Thus, TOXCAT is a convenient screening method to measure the strength of self association between TM helices.

To map its interaction interface, site-directed mutagenesis is introduced to ADCK3 to study the role of different amino acids in the dimerization of the TM domains, and its corresponding CAT activity is then compared to that in the wild type (WT) TM peptide. The CAT activity of each mutated TM α-helices is compared to that of the wild type and the interaction interfacial residues are thereby identified by the mutants that disrupt the CAT activity compared to the WT sample.

METHODS

Site-directed Mutagenesis to Create TM Region of Interest

Site-directed mutagenesis was carried out to generate the mutated transmembrane gene of interest in the pcckan vector. Primers with sequence of gene of interest were designed and purchased from the Integrated DNA Technologies (IDT). The oligo used for mutant L214I is represented as followed:

Forward Primer: 5' CCA AAC TTG GGG AAT CGA GCT AGC ATC GCC AAC TTC GGA GGT CTG GC 3'
Reverse Primer : 5' GCC AGA CCT CCG AAG TTG GCG ATG CTA GCT CGA TTC CCC AAG TTT GG 3'

Each reaction contains 1.25 U of DNA polymerase, 1xPfu buffer, 0.2mM of dNTP, ADCK3 DNA template, and 10uM of the forward and reverse primers. A total of 53 reactions, including a positive control of WT ADCK3 were prepared. All reactions were run in thermocycler overnight for a total of 30 cycles. An example of the site-directed mutagenesis of L214I from Leucine ('L') to Isoleucine ('I') at position 214 is represented as followed:

Wild Type: " **214 LANFGGLAVGLGFGALA 231**"

Mutated : " **214 IANFGGLAVGLGFGALA 231**"

The underlined region is the TM region of interest and it is flanked by pcckan vector.

Transformation into DH5α cells and DNA Purification

The construct was consequently transformed into DH5α cells via electroporation. Cells were grown in Petri dish rich in lysogeny broth (LB) agar. From the overnight grown plate, one fresh colony was selected and inoculated into 3 mL of LB broth containing 100μg/mL of

ampicilin. Plasmid DNA extraction technique (miniprep) was performed to purify plasmid DNA from bacteria with miniprep kit purchased from Qiagen. Big dye reaction with pcckan specific primer was carried out to amplify the DNA sequence before the DNA sequence was sent to the UW sequencing facility at the University of Wisconsin Biotech Center for sequencing. After the mutated sequence of gene of interest was confirmed, the construct was transformed into MM39 cells and TOXCAT assay was carried out to determine the level of dimerization in both the wild type and mutated TM region of *E.coli*. In order to investigate if the construct is properly inserted into the membrane region, the mutants were transformed into Maltose plate.

If the construct is properly inserted, it shows positive results as shown in Figure 2.

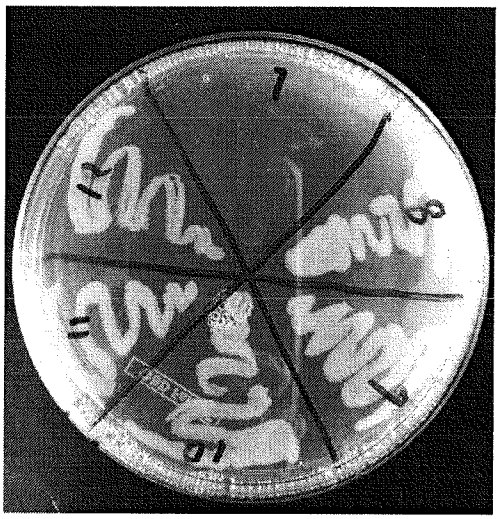


Figure 2. The correct insertion of the TM region is confirmed by growth on minimal media with maltose as the carbon source. The construct growth in Maltose media represents the TM is properly inserted into the TM region. Construct L214F (marked as 7) did not grow in Petri dish rich in Maltose media, and it represents the TM region is perhaps not properly inserted.

Transformation into MM39 cells for Expression and TOXCAT Assay

TOXCAT constructs that contain the mutated gene of interest were transformed into the MM39 cells for expression. A fresh colony was inoculated into 3 mL of LB broth with ampicillin and grown overnight at 37 °C. From this seed culture, 30 uL of bacteria culture were inoculated

into a fresh 3 ml LB tube with ampicillin and grown at 37 °C for about 2 hours. After checking the optical density at 420 nm to be between 0.8 and 1.1, 1 mL of cells were spun down at 17000g for 10 minutes. The supernatant was removed and the cell pellets was resuspended in 500 μL of sonication buffer consisted of 25mM of Tris-HCl and 2mM of EDTA at pH8.0. Sonication was carried out in the sonicator for 8 seconds to break the cellular membrane and then spun at 17000g for 10 minutes. The lysate obtained was then used for the spectrophotometric CAT assay for measuring the dimerization extent.

The level of dimerization of TM protein or CAT activity was monitored via UV/Vis spectrophotometry. 1 mL of reaction buffer (0.1mM acetyl coA, 0.4 mg/mL Ellman's reagent, 0.1 M Tris-HCl, pH 7.8) was prepared and mixed with 40 μL of lysate sample. The background signal at 412nm was recorded for 1.5 minutes and 40 μL of 2.5 mM chloramphenicol was then added. The absorbance at 412 nm was continued to be measured for additional 1.5 minutes. All the measurements were performed in duplicates and the average was calculated. The CAT activity in wild type was compared to that of the mutated TM domains and the activity was calculated as followed:

$$\textbf{Relative CAT activity} : \frac{\textbf{(Slope}_{412nm} \textbf{- background) x 100}}{\textbf{Cell Density (OD}_{420nm})}$$

The slope of the curve generated at 412 nm after chloramphenicol addition was subtracted from the slope of the background absorbance at 412 nm and divided by the optical density at 420nm (OD_{420nm}) of the cells. CAT activity of the mutants was then normalized with the CAT activity in the ADCK3-WT.

RESULTS & DISCUSSION

Comparison of CAT Activities in WT-ADCK3 and Mutants

In order to map the interaction interface of ADCK3, site-directed mutagenesis was performed to demonstrate the positions that are important for association. A total of 53 mutants were generated and analyzed via the TOXCAT assay. A variety of hydrophobic amino acids were substituted at each position. The relative CAT activity of each mutant relative to the ADCK3-WT was shown in Figure 3. The average disruption of each mutant at every position was classified into four categories, "WT-like" (>80% WT CAT activity), "Mild" (50-80%), "Severe" (20-50%) and "Disruptive" (0-20%) as shown in dashed lines in Figure 3 (Khadria et al., *manuscript in preparation*)

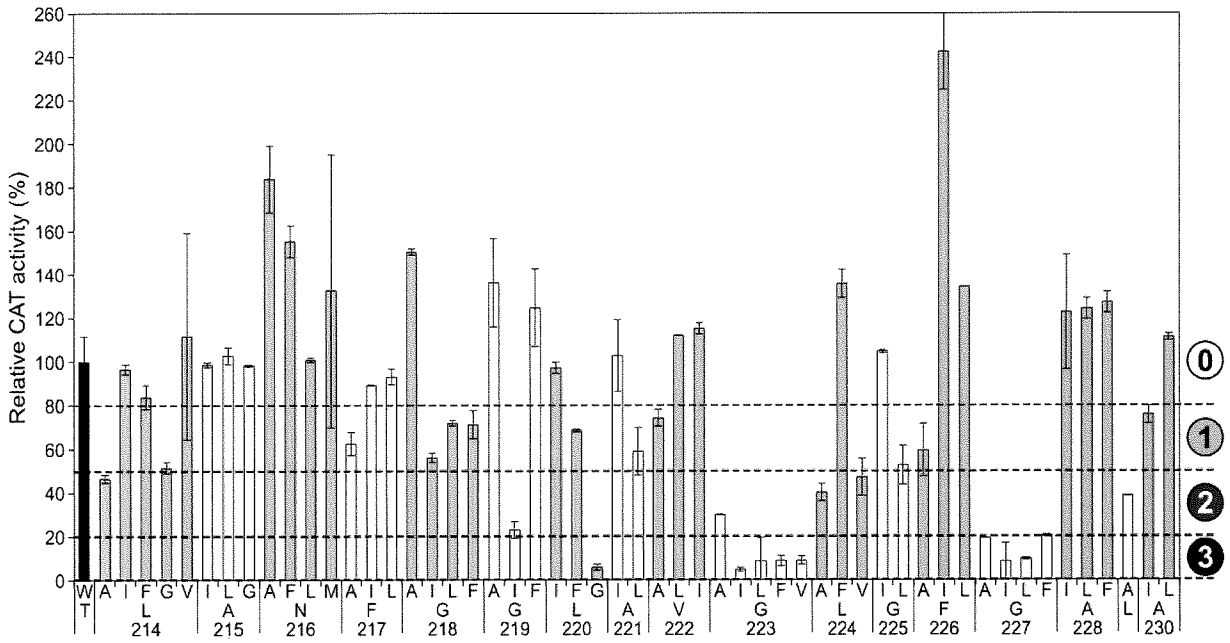


Figure 3. TOXCAT data of all the mutations on ADCK3-TM measured via TOXCAT assay. The substitution of glycine residues at positions 223 and 227 disrupts the helix-helix association of the TM, thus it shows lower CAT activities. The relative CAT activities of mutants at positions 223 and 227 are about 10-20% compared to ADCK3-WT. The substitution at both positions interrupts the GxxxG motif that subsequently affects the association of α-helices in the TM region of ADCK3. (Figure extracted from Khadria et al., *manuscript in preparation*)

ADCK3-WT, (shown in black at 100% CAT activity in Figure 3) represents a strong homodimer in the TM of *E. coli*. The error bars shown are values obtained from the standard deviation of mutants analyzed relative to the WT. The relative dimerization of mutants varies from highly disruptive to stability greater than the wild type. In the TM domains of ADCK3, we carried out substitution by changing the size of the amino acids one by one. Depending on whether the position being mutated is at the dimerization interface, there is either a disruption of dimerization or not. Certain outliers with high standard deviation are possibly experimental errors and need to be repeated. Mutants that show negative signals are not included in the experimental data shown. Samples with negative signals usually represent certain mutants that do not grow in Maltose media, indicating that perhaps the construct is not properly inserted in the membrane.

Our experimental data also depicts the sensitivity of mutants at various positions. L214F represents the mutation of leucine residue to isoleucine residue at position 214. The resulting CAT activity does not vary much compared to the WT. In addition, valine is replaced by leucine and isoleucine at position 222, and the resulting CAT activities show similarity to that in wild type. This suggests that positions 214 and 222 are not important in forming the TM dimer.

"MacKenzie Plot" to Summarize the Degree of Disruption in TM of ADCK3

The average disruption of each mutant was also depicted in "MacKenzie plot" as shown in Figure 4a. It shows the effect of each mutant at a particular position. The average disruption was divided into a scale of zero to three, from "WT-like" to "Disruptive". The substitution of glycine residues at positions 223 and 227 is highly disruptive to the TM of ADCK3. The average disruption at each position was also plotted numerically in Figure 4b.

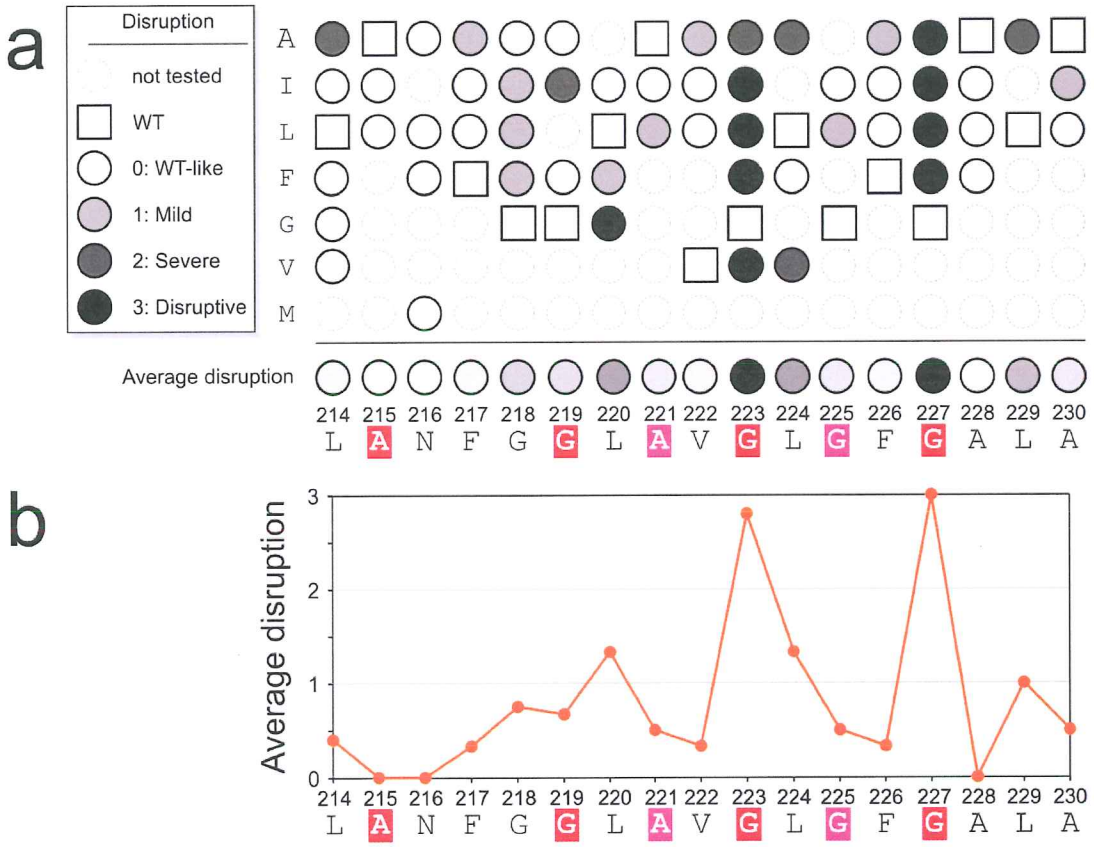


Figure 4. The importance of positions 223 and 227 in association. a) "MacKenzie plot" is used to show the degree of disruption in the TM of ADCK3-WT. A scale of 0-3, from 'WT-like' to most disruptive was assigned to show its relative stability compared to the WT. **b)** A numerical plot of the relative stability was created. Mutants at position 223 and 227 show the highest average disruption. (Figure extracted from Khadria et al., *manuscript in preparation*)

G^{223} xxxG^{227} or GxxxG-like Motifs increase TM stability

The GxxxG motif, a signature found in TM regions, responsible of homodimerization of the proteins (Senes et al., 2004). The naturally occurring GxxxG motif is seen frequently with large residues such as valine, leucine, and isoleucine at the position neighboring to the glycine residues (Russ, Engelman, 2000). The GxxxG-like motif is another in which one or both glycine residues are substituted by another small residue, such as alanine or serine. ErbB receptor is one of the biological instances that have GxxxG motif. It has two conserved GxxxG-like motifs at the N- and C- terminal ends, one responsible for homodimerization and the other for heterodimerization with another subunit of the ErbB receptor (Senes et al., 2004). In addition, GxxxG motif can also be seen in F_0F_1-ATP synthase. A study performed by Arselin et al. (2003) shows that the substitution of glycine to another residue impairs the ability of the ATP synthase to form oligomeric complexes.

In ADCK3, glycine residues at positions 223 and 227 represents the GxxxG motif. The mutations of G223F and G227F are particularly interesting. As shown in the graph, the substitution of glycine to phenylalanine at positions 223 and 227 disrupts the dimerization of TM domains and decreases its CAT activity level to about 10~20% of WT. The insertion of another amino acid residue to the GxxxG motif has interrupted its conformation, and it affects the α-helices association in ADCK3. The substitution of phenylalanine at positions 223 and 227 disrupts the homodimer, and the observation suggests that both positions play a key role in dimerization of TM proteins.

Formation of the ADCK3 Homodimers as Predicted By CATM

In order to understand the structural aspect of the homo-oligomerization of the TM of ADCK3, an analysis via CATM was performed. CATM is a program used to predict the structure of transmembrane homodimers mediated by the GxxxG motif (Mueller et al., 2014).

As shown in Figure 5, five possible models of the formation of helices in ADCK3 were generated by CATM. Table 1 below shows the geometry of the five models. The position of association was also highlighted and marked as 'dot' as shown on the amino acid sequence of each model in Figure 5. The crossing point of the association was marked and shown in the green parallelogram.

	d (Å)	θ (°)	*Z'* (Å)	ω' (°)	*E*
Model 1	6.6	-27.1	4.2	51.9	-59.8
Model 2	6.3	-41.2	0.6	42.8	-50.8
Model 3	6.3	-44.0	0.9	46.1	-47.7
Model 4	6.7	-49.9	2.8	47.0	-31.4
Model 5	6.9	-25.0	4.8	50.3	-29.6

Table 1. CATM predicts the geometry of the five models. The symbols are represented as followed: interfacial positions distance (d); crossing angle (θ); vertical (Z') and axial (ω') coordinates of the crossing point within the parallelogram of closest approach; and energy score E. (Figure extracted from Khadria et al., *manuscript in preparation*)

The crossing angle of model 4 is the highest while the crossing angle of model 1 is the lowest. From Figure 5, the helices of Model 2 cross at glycine residue at position 223 which is compatible with our experimental data. The crossing angle of approximately 40^0 is also ideal for the formation of the TM helices (Mueller et al., 2014).

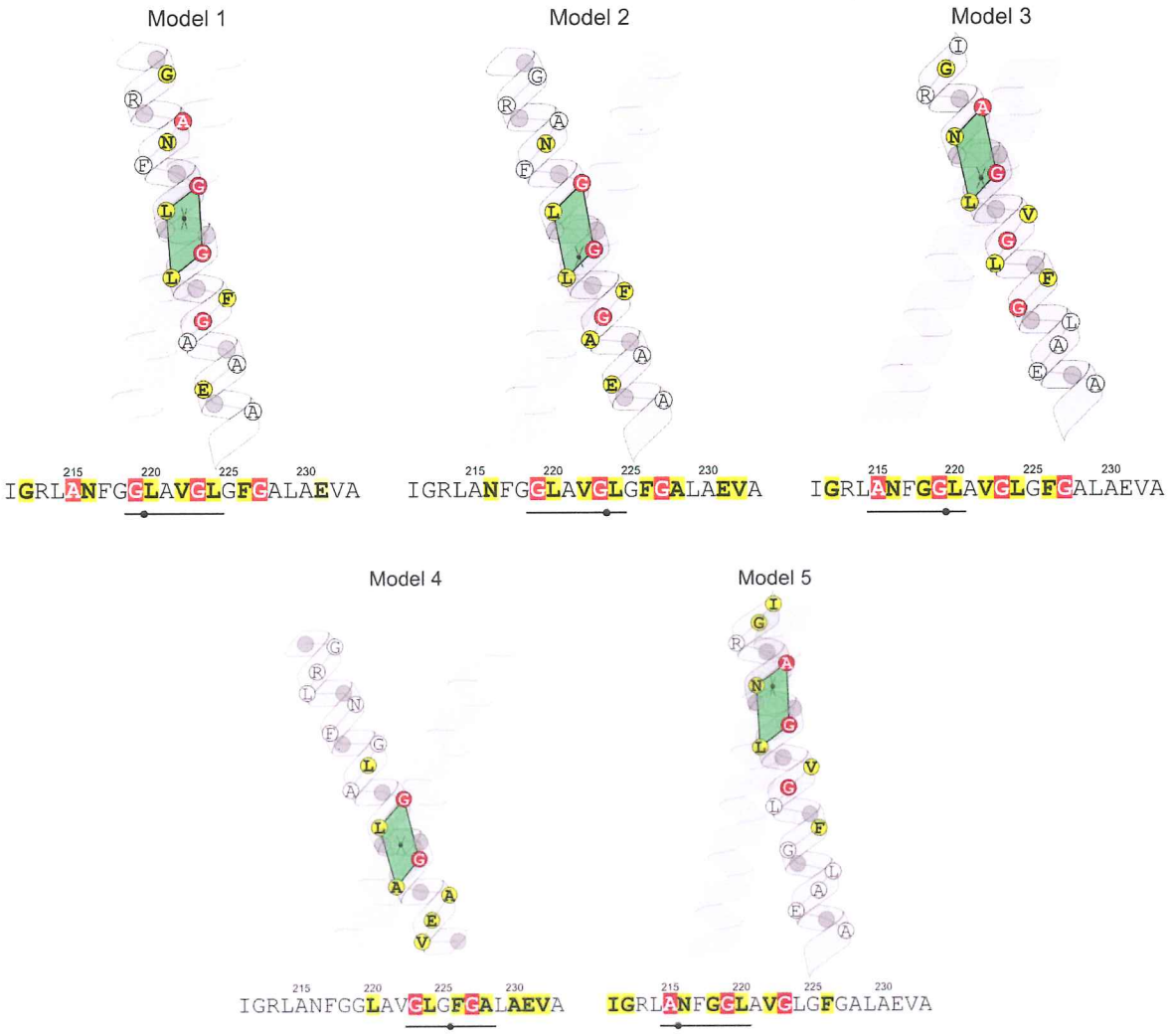
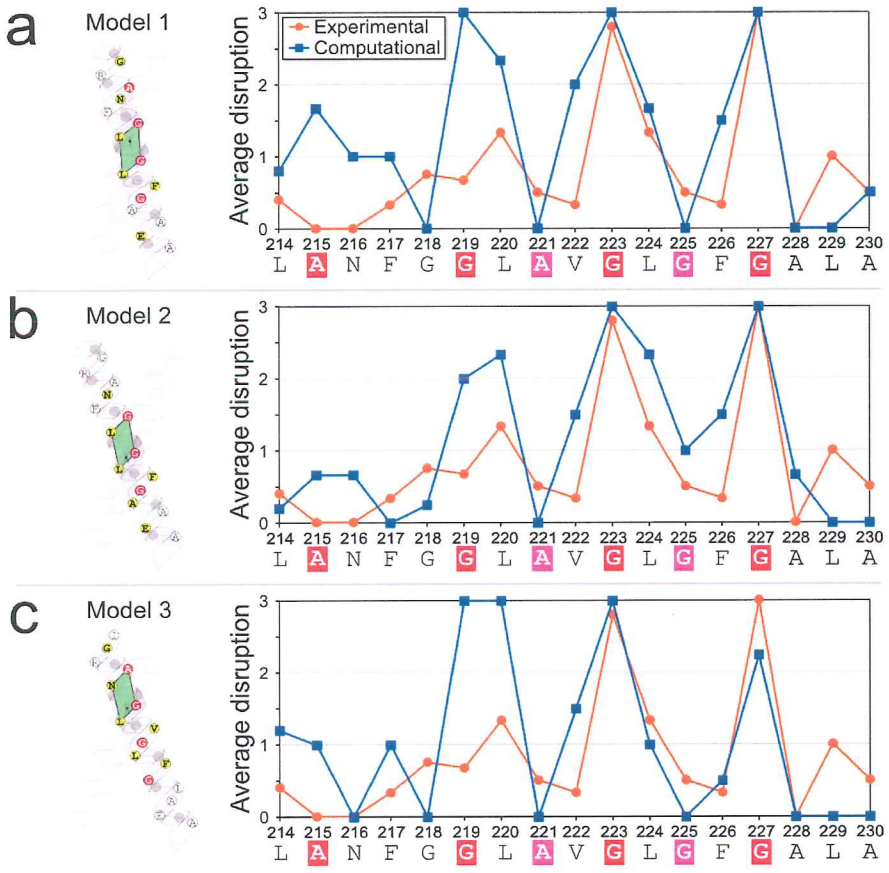


Figure 5. CATM depicts the geometrical features of the homodimer formed in the TM of ADCK3. Model 2 shows the crossing angle of the helices at positions 223, which aligns to the positions of the formation of the GxxxG motif found in the TM region of ADCK3. (Figure extracted from Khadria et al., *manuscript in preparation*)

Comparison of Mutagenesis Data in TOXCAT and Computational Modeling

In order to investigate the consistency of the data from the TOXCAT assay and computational analysis from CATM, a graph of the disruption pattern of each model was generated as shown in Figure 6. The orange lines correspond to the experimental data from TOXCAT assay whereas the blue lines correspond to the computational data from CATM.

The data from the TOXCAT assay shows that the positions 223 and 227 are highly sensitive to mutation. Models 1, 3, and 5 show highly disruptive at position 219, which does not match with our experimental observations.



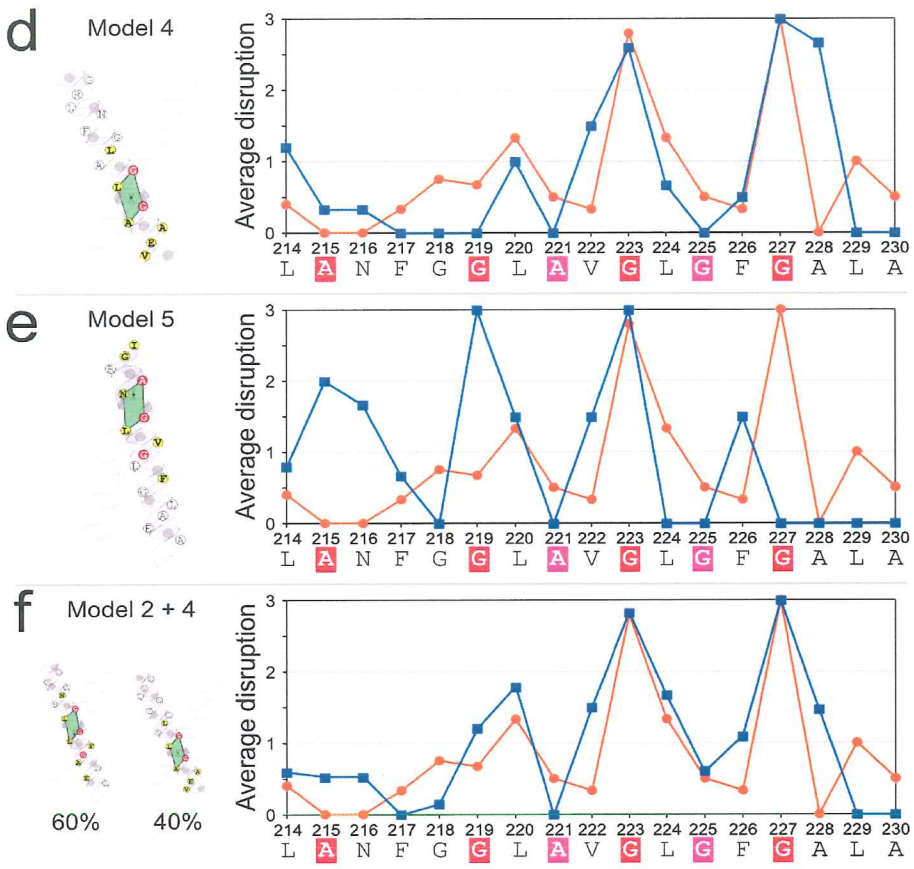


Figure 6. Comparison of the mutagenesis data in TOXCAT and computational modeling. Model 2 represents the best structure for the formation of helices in the TM region of ADCK3 with average disruption peaks at positions 223 and 227, the positions of the formation of the GxxxG motif. (Figure extracted from Khadria et al., *manuscript in preparation*)

Models 2 and 4 are better structure predictions of the association of ADCK3. They peak at positions 223 and 227 which is in agreement with our experimental data that the mutations at positions 223 and 227 disrupt the GxxxG motif. However, model 4 shows a peak at position 228 as shown from the analysis of CATM. This does not agree with our experimental data. Therefore, the observations from TOXCAT and CATM conclude that model 2 is the best geometrical model of the helix-helix association of the TM of ADCK3.

The Role of Cα-H Hydrogen Bonds in TM Association

Hydrogen bonds play a crucial role in proteins stability and folding (Senes et al., 2001). Popot et al. have shown the importance of hydrogen bonds in an apolar environment in stabilizing the helical conformation of TM. The Cα-H···O=C bonds is frequently found in the glycophorin A (GpA) and helices containing the GxxxG motif (Senes et al., 2001). The main feature of GxxxG motif is that it has been optimized for carbon hydrogen bonds between Cα-H donors and backbone carbonyl oxygen acceptors (Mueller et al., 2014). The alpha carbon acts as an electron donor and the carbonyl carbon accepts the electron. Studies done by Mueller et al. have also shown that the electron withdrawing amide N-H groups help to overcome the elctropositivity of the α-carbon atom.

The presence of Cα-H···O=C bonds is hypothesized to be the main driving force for the interaction of TM helices. Small amino acids such as alanine or serine help to reduce the steric hindrance to allow the interaction of helices to form the Cα-H···O=C bonds. In GpA, studies done by Walters and DeGrado have shown that the crossing angle of the right-handed α-helices is approximately 40°. The crossing angle of 40° is ideal for these interactions to contribute to helix-helix formation. However, more studies have to be done on the thermodynamics and kinetics of hydrogen bonds in the TM region to understand the contribution of each aspect to the TM helices association.

Figure 7 shows that the glycine residue has a hydrogen atom as its side chain and it forms the Cα-H hydrogen bonds with another glycine residue to form the homodimer (Senes et al., 2001).

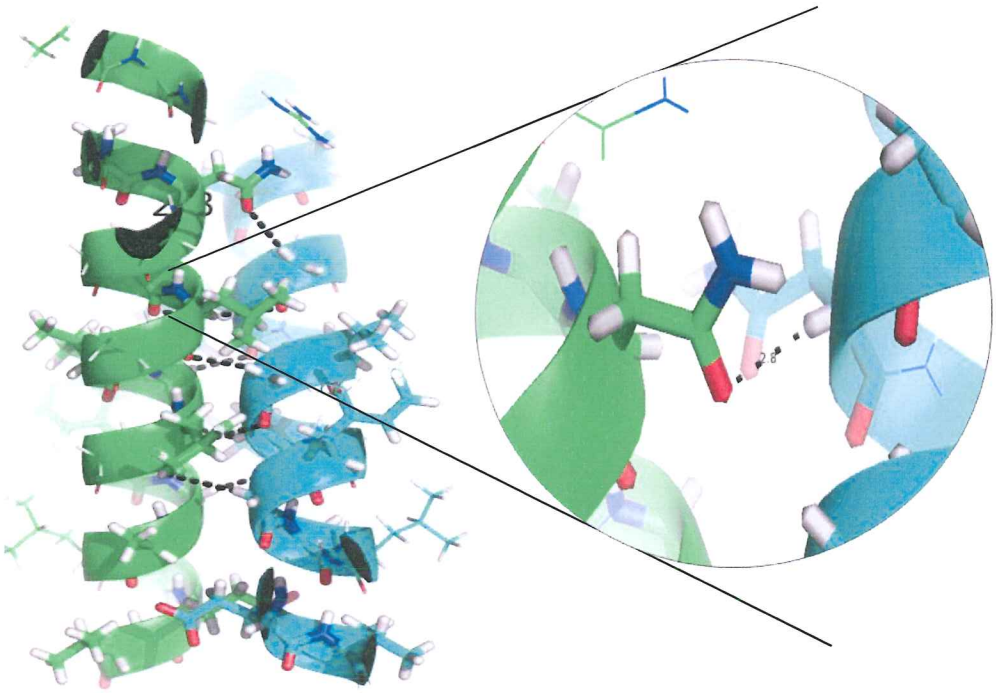


Figure 7. The formation of alpha Cα-H hydrogen bonds. The glycine residue has a hydrogen atom as its side chain and it forms a alpha Cα-H hydrogen bond with another glycine residue to form the homodimer. The TM helix-helix associated is mediated by GxxxG motif, a signature motif that responsible for the dimerization of TM region of ADCK3.

CONCLUSIONS

The glycine residues at positions 223 and 227 form the GxxxG motif, a signature motif frequently found in TM. The GxxxG motif is crucial for the TM helices association in ADCK3. The mutations at both positions decrease the CAT activity to 20-30% of the WT. The formation of the alpha Cα-H hydrogen bond drives the formation of homodimer in the TM region of ADCK3. CATM predicts the structure of ADCK3 with multiple conformations. Analysis from CATM shows that Model 2 matches with our experimental data from the TOXCAT assay.

Further studies are being carried out to include more mutations to map the interaction interface of ADCK3 using the TOXCAT assay. In addition, the mutants that do not grow in maltose media and show negative signals have to be repeated. One of the plausible reasons is that the construct is not properly inserted in the membrane.

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