RESEARCH ARTICLE

S6 peptide derived from KvAP channel shows marked differences in secondary structure after mutation- a computational approach

Chetan Malik[1]*, Rajan Shrivastava[2], Subhendu Ghosh[2], Hasan SS[1]

[1]Department of Life Science, School of Sciences, Indira Gandhi National Open University, New Delhi, 110068, India
[2]Department of Biophysics, University of Delhi South Campus, New Delhi, 110021, India
*Corresponding author: Email: dfinechetan@gmail.com | Phone +919818994921

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ABSTRACT

KvAP is a widely studied potassium channel from bacterium *Aeropyrum pernix.* It is composed of six trans-membrane domains ranging from S1 to S6. Out of these six domains, S6 is the domain through which transfer of ions occurs. We have analyzed the structural differences in the s6 domains after mutating more hydrophobic amino acids with less hydrophobic amino acids. The computational studies suggest that there is a change in the secondary structures of protein which leads to its altered functioning. The RMSD (Root mean square distance) of the peptides were calculated and significant differences were observed in Ramachandran plot. The computational analysis complements the earlier published results.

Keywords: KvAP channel, Secondary structure, Ramachandran plot, RMSD (Root Mean Square Deviation), Hydrophobicity.

INTRODUCTION

KvAP is a voltage gated potassium channel found in bacterium *Aeropyrum pernix* (Cohen, Grabe, & Jan, 2003; Malik & Ghosh, 2013; Verma et al., 2011). It is widely studied channel which explains the fundamental properties of a potassium channel (Cohen et al., 2003; Lee, Lee, Chen, & MacKinnon, 2005). Principally, it consists of six trans-membrane domains named S1 to S6. These domains organize themselves in a tetramer assembly to form the functional potassium channel. S1 to S4 lies on the peripheral boundary while S5 and S6 form the pore domain (Cuello, Cortes, & Perozo, 2004; Jiang, Wang, & MacKinnon, 2004). Out of S5 and S6, the later form the pore wall. S6 is the domain through which permeation of the ions take place (Cuello et al., 2004; Jiang et al., 2004).

People have done mutation in the ion conduction pathway of potassium channels and other ion channel to study their functioning (Vornanen, 2005). There are certain regions in the pore domains that are critical to functioning of the ion channels. Generally, people mutate those regions

that are evolutionary conserved. Glycine is one of the amino acid which is not only conserved in prokaryotes but also in the eukaryotes (Swartz, 2005). Glycine being the simplest amino acid provides space and flexibility to the movement and helps in gating of the channel. Earlier we have synthesized 22 amino acid peptide from pore domain i.e. S6 containing glycine residue and mutated it with other residues and considerable differences were observed in the Circular dichroism spectra (Verma et al., 2011). The purpose of this communication is to explore the sequence specific role of pore domain by mutating certain portion of S6 peptide and see the structural and functional differences through computational studies. We have performed the sequence alignment of the peptide and its mutants and calculated the RMSD value. Further Ramachadran plots were drawn for these peptides. We have found that the secondary structure of these peptides differs a lot and these differences are critical to the functioning of the ion channel. The computational studies complement the structural and functional studies performed by us in our previous communications (Malik & Ghosh, 2013; Verma et al., 2011).

MATERIALS AND METHODS

1. Peptide sequences:
We have selected 22 amino acids of S6 domain of KvAP channel and mutations were done in the sequence to create new peptide and named as S6 SCR and S6M. The peptides were synthesized by the method as described by Malik and Ghosh 2013 and Verma *et al* 2011(Malik & Ghosh, 2013; Verma et al., 2011). The structure of peptides is as follows:-

S6- LTGISALTLLIGTVSNMFQKIL
S6M- LTGISALTLL***GI***TVSNMFQKIL
S6SCR- LTG***ASI***LTLL***GI***TVSNMFQKIL

2. Analysis of structure in software MPeX (Membrane Protein explorer):
The structure of the peptides was analyzed in software MPeX (Membrane protein explorer) developed by Department of Physiology and Biophysics in the School of Medicine of the University of California at Irvine (Snider, Jayasinghe, Hristova, & White, 2009). Earlier the circular dichroism spectra showed the differences in the %age of helicity of three peptides (Verma et al., 2011); which are as follows: - S6 ~19 %, S6SCR~15%

and S6M~32 % (S6M; Unpublished results). Based on the differences in the helicity of these three peptides, we have calculated the free energy through totalizer package of MPeX software and plotted its helical wheel projection. The MPeX package takes care of the experiments done in the bilayer electrophysiology as it considers the environment of lipid to calculate the free energy (Ladokhin & White, 1999). Earlier, Bilayer electrophysiology experiments were done in buffer lipid partitioning therefore we used the same for simulation in MPeX software.

3. Prediction of structure and creation of model pdb through iTASSER:
The primary sequence of peptides were uploaded to iTASSER website (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) which gives us the predicted structures of the three peptides along with pdb files (Roy, Kucukural, & Zhang, 2010; Yang et al., 2015; Yang & Zhang, 2015; Zhang, 2008).

4. Prediction and analysis of Ramachandran plots:
The Ramachandran plots were drawn from the website Molprobity [http://molprobity.biochem.duke.edu/].
Ramachandran plots are drawn based on the phi-psy angles. It gives the differences in the secondary structures of peptides on mutation. For plotting the Ramachandran graphs we have uploaded the pdb files of all the peptides on the website. Considerable differences were observed in the Ramachandran plots which have been discussed in the later section.

5. Calculation of RMSD:
For calculation of RMSD of the peptides, we have used pymol software [https://www.pymol.org/]. First of all the pdb files of the peptides were loaded in the pymol and structure of S6SCR was aligned to S6. Cealign command prompt gives us the differences in the structure (RMSD) of the two peptides. Similarly, RMSD of S6M was calculated and compared to native peptide i.e. S6.

6. Comparison of secondary structure in software VMD (Visual Molecular Dynamics):
The pdb files of three peptides were loaded into VMD software (Humphrey, Dalke, & Schulten, 1996; Roberts, Eargle, Wright, & Luthey-Schulten, 2006). VMD software has inbuilt tool known as Multiseq

which create the multi-sequence alignment of peptides/ proteins and on the basis of multi-sequence alignment the programme gives the Qres score which is nothing but the sequence conservation score. Other useful feature of VMD is that it gives us the RMDS value of each residue. It plots the RMSD values of each residue for the peptide. By doing this we can compare the secondary structure difference of each peptide and construct the models/phylogenetic trees.

RESULTS AND DISCUSSION

For comparison of the secondary structures of three peptides, modeling of these peptides was done by using i-TASSER software suite. It generates different pdb files and gives them different scores that are also known as C-Score. C score is confidence score which is the rough estimate of predicted model. The range of C

score is in between -5 to +2. A higher value of C score means that the predicted model has high confidence and the reverse is also true. TM score and RMSD is calculated based on known structure i.e. KvAP template. As per i-TASSER, TM score is estimated to overcome the problem in calculation of RMSD value as RMSD value is the average of deviation considering all big and small difference of individual amino acids between two proteins (Zhang & Skolnick, 2004). A TM-score >0.5 means that predicted model has good topology where as a TM-score <0.17 means that there is random similarity between two proteins. Table 1 gives us the C-score, TM score and estimated RMSD values of the most correct model predicted by i-TASSER. We find from the table 1 that the C-score is considerably good and TM value of the peptides is also >0.6. However, there are considerable differences in the RMSD values of these models. Hence we have used these pdb files of these models for further analysis.

Table 1.

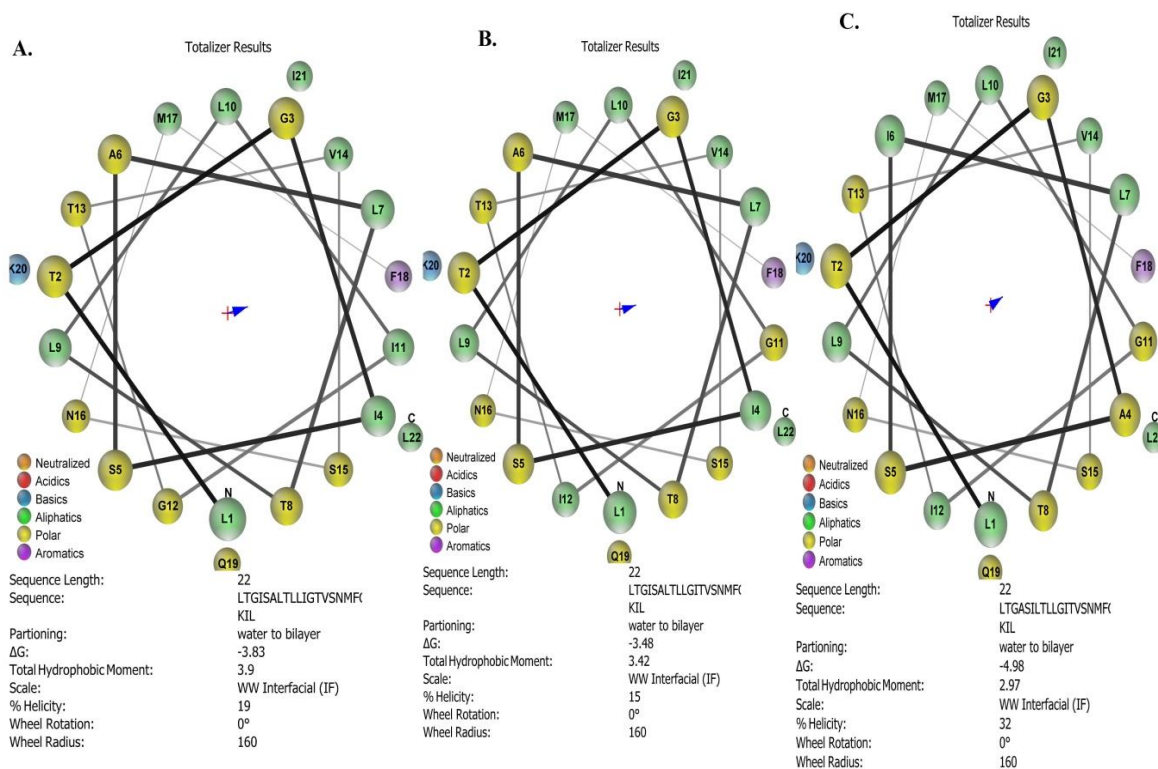| Peptide	C-Score	Estimated TM-Score	Estimated RMSD
S6	-0.30	0.67±0.12	1.7±1.5Å
S6M	-0.67	0.63±0.14	2.4±1.8Å
S6SCR	-0.39	0.66±0.13	1.9±1.5Å

Figure 1: Helical wheel projection of S6 peptide and its analogs;
A. S6 at %age helicity of 19%, B S6M at %age helicity of 15% and C S6-SCR at %age helicity of 32%. The negative values of ΔG suggest that they have considerable amount of free energy to form the stable structure inside the membrane. Total hydrophobic moments follow the order S6SCR<S6M<S6, showing S6SCR activity to be the lowest among all.
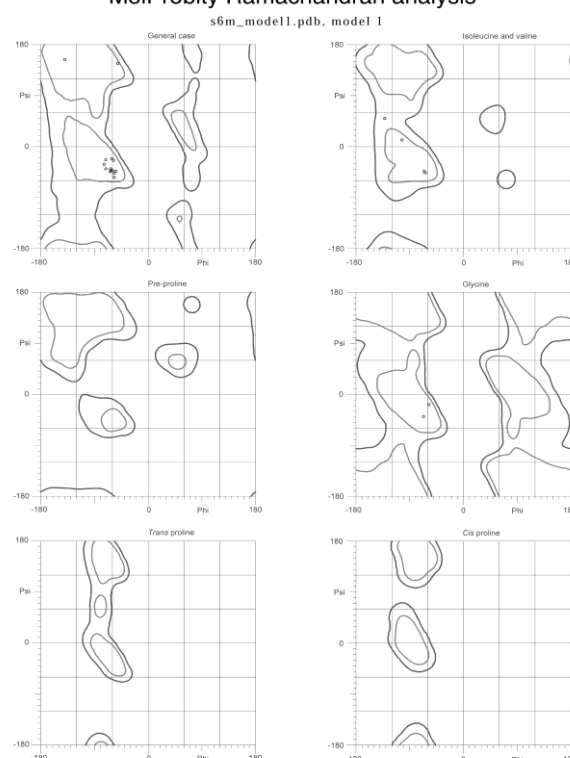
MolProbity Ramachandran analysis
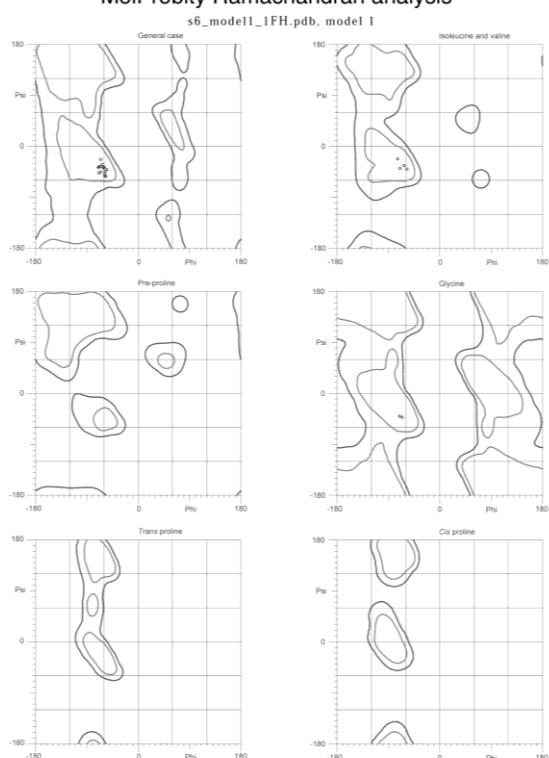
s6_model1_1FH.pdb, model 1

MolProbity Ramachandran analysis

s6m_model1.pdb, model 1

100.0% (20/20) of all residues were in favored (98%) regions.
100.0% (20/20) of all residues were in allowed (>99.8%) regions.
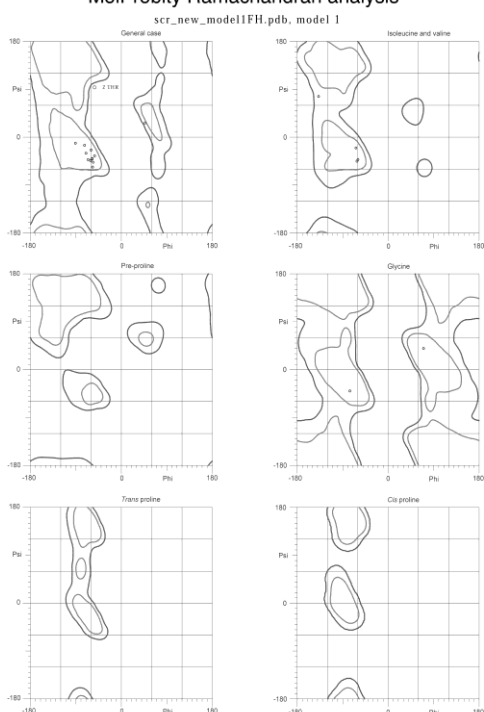
There were no outliers.

95.0% (19/20) of all residues were in favored (98%) regions.
100.0% (20/20) of all residues were in allowed (>99.8%) regions.

There were no outliers.

MolProbity Ramachandran analysis

scr_new_model1FH.pdb, model 1

85.0% (17/20) of all residues were in favored (98%) regions.
95.0% (19/20) of all residues were in allowed (>99.8%) regions.

There were 1 outliers (phi, psi):
   2 THR (-53.9, 94.4)

Figure 2: The Ramachandran plots for S6 peptides and its analogs;
A. S6 showing 100% residues in the favoured and allowed region with no residue in outlier region, B. S6M showing 95% & 100% residues in the favored and allowed region respectively with no residue in the outlier region, C. S6SCRS6M showing 85% &95% residues in the favored and allowed region respectively with one residue i.e. Threonine of position two of the given sequence in the outlier region. The plots show that S6 is the most stable structure among all with all the angles in the favourable region with no bad stoichiometry. The order of peptide according to good structure and stoichiometry is S6SCR<S6M<S6 confirming the earlier results.

Table 2 :Table showing the overall result analysis of Ramachandran plots for S6 peptide and its analogs;

A.

All-Atom Contacts	Clashscore, all atoms:	0		100th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	0	0.00%	Goal: <0.3%
	Favored rotamers	19	100.00%	Goal: >98%
	Ramachandran outliers	0	0.00%	Goal: <0.05%
	Ramachandran favored	20	100.00%	Goal: >98%
	MolProbity score^	0.50		100th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 162	0.00%	Goal: 0%
	Bad angles:	0 / 218	0.00%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 0	0.00%	Expected: ≤1 per chain, or ≤5%

B.

All-Atom Contacts	Clashscore, all atoms:	0		100th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	0	0.00%	Goal: <0.3%
	Favored rotamers	19	100.00%	Goal: >98%
	Ramachandran outliers	0	0.00%	Goal: <0.05%
	Ramachandran favored	19	95.00%	Goal: >98%
	MolProbity score^	0.85		100th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 162	0.00%	Goal: 0%
	Bad angles:	1 / 218	0.46%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 0	0.00%	Expected: ≤1 per chain, or ≤5%

C.

All-Atom Contacts	Clashscore, all atoms:	0		100th percentile* (N=1784, all resolutions)
	Clashscore is the number of serious steric overlaps (> 0.4 Å) per 1000 atoms.			
Protein Geometry	Poor rotamers	0	0.00%	Goal: <0.3%
	Favored rotamers	19	100.00%	Goal: >98%
	Ramachandran outliers	1	5.00%	Goal: <0.05%
	Ramachandran favored	17	85.00%	Goal: >98%
	MolProbity score^	1.16		99th percentile* (N=27675, 0Å - 99Å)
	Cβ deviations >0.25Å	0	0.00%	Goal: 0
	Bad bonds:	0 / 162	0.00%	Goal: 0%
	Bad angles:	1 / 218	0.46%	Goal: <0.1%
Peptide Omegas	Cis Prolines:	0 / 0	0.00%	Expected: ≤1 per chain, or ≤5%

A. analysis of S6 results, **B**. analysis of S6M results and **C**. analysis of S6-SCR results.
The green color highlights the allowed stoichiometry while yellow and pink shows the critical values. As evident from the table S6 structure is most stable amongst all with no bad stoichiometry as mentioned earlier.

In our earlier papers, we calculated the percentage helicity of S6 and S6-SCR to be 19% and 15% respectively through Circular dichroism (Verma et al., 2011). Later we calculated the percentage helicity of S6M to be 34% and confirm the result of percentage helicity of S6 as mentioned earlier. The experimental data were used to calculate the ΔG i.e. free energy of the three peptides. All the three peptides showed negative free energies (Figure 1A-C). This means that the peptides will form channels in the lipid bilayer. Using bilayer electrophysiology technique, we have characterized the activity of three peptides. We have

shown S6 and S6-SCR form channels in the membrane but S6 was comparatively more active than S6SCR due to higher helicity (19% compared to 14%). Figure 1 A, B and C shows the hydrophobic moment of the S6, S6M and S6SCR respectively, by calculating through MPeX software which follows the order S6-SCR<S6<S6M and hence we can conclude that S6SCR should be less active in the membranous environment which was found to be experimentally true (Verma et al., 2011).

Ramachandran plots give us the analysis of secondary structure of peptide by plotting phi-psi angles and calculate the geometrical favorability of the structure. Figure 2 A, B & C shows the Ramachandran plots of S6, S6M and S6SCR respectively. The analysis of the Ramachandran plots has been shown in Table 2A (S6), B (S6M) and C (S6SCR). We have found S6to show the most stable and favorable structure with no outliers, as there is no bad angles present in the structure. All the atoms lie in the favorable region. On the other hand when we compare S6M with S6, although there are no outliers but 95% of the atoms lie in the favourable region. However, there is presence of one bad angle. When S6SCR is compared to S6, there is one outlier that is threonine located at the position 2 in the S6-SCR sequence. It has phi angle as $\sim -54^0$ and psi angle as $\sim 94^0$. The table gives us the stability profile of the structure. 85% of the residue are in the allowed region which should be >98%. 95% of the structure is favoured instead of 100%.

In the figure 3 we have predicted the secondary structure of S6 peptide and its analogs using software Pymol (Figure 3 A. S6 (Green), B. S6M (Blue) and C. S6SCR (Magenta). The figure depicts the altering of the more hydrophobic amino acids with less hydrophobic one (Isoleucine with alanine in S6SCR and isoleucine with glycine in both S6M and S6SCR) which changes the percentage helicity of the peptides.

The Q_{res} determines the structural homology among proteins to be compared. The maximum value of Q=1 which means structures to be identical. A low score means the value between 0.1 and 0.3, structures are not well aligned. This further indicates that only few carbon atoms in the structure may superimpose on each other. Similarly, with RMSD and relative sequence conservation, speaks about how predicted structures are aligned to each other. The pdb files generated earlier were transported to software VMD for calculation of Q_{res} score, RMSD per residue and

relative sequence conservation (Humphrey et al., 1996). The result of the analysis has been given in figure 4 A, B and C respectively. Figure 4A suggests that the value of Q_{res} for S6 is always higher than 0.75 this means S6 has stable structure and pretty well aligned. The value of Q_{res} falls less than 0.7 for 3rd and 4th amino acid for S6M. Similarly, in case of S6SCR the value of Q_{res} is less than 0.65 for 4th, 5thand 6th amino acid. Mutation has been done exactly at this place. Figure 4B gives the RMSD value per residue of these peptides. As evident from the figure, S6M has highest variation of this value for first 4 amino acids while it has highest variation for first six amino acids in case of S6SCR. Figure 4C shows the relative structural conservation. As shown in figure S6SCR is more diverse than S6M till first six amino acids. Although variation also exists beyond 6th amino acids for these two peptides but they are not altering the Q_{res} value and RMSD values. The stoichiometric differences occurring due to mutation is causing S6SCR to become less active.

S6 is the innermost domain of KvAP channel which is known to form the hydrophobic inner core for transfer of ions from one side of the membrane to the other (Cohen et al., 2003; Cuello et al., 2004; Swartz, 2005). Moreover it contains various evolutionary conserved amino acids (Blunck & Batulan, 2012; Labro & Snyders, 2012). One of such amino acids is Glycine. Because of simplest structure of this particular amino acid; ion-channel is able to bend properly and hence it helps in conduction of ions because of opening and closing of the channel (often referred as gating).
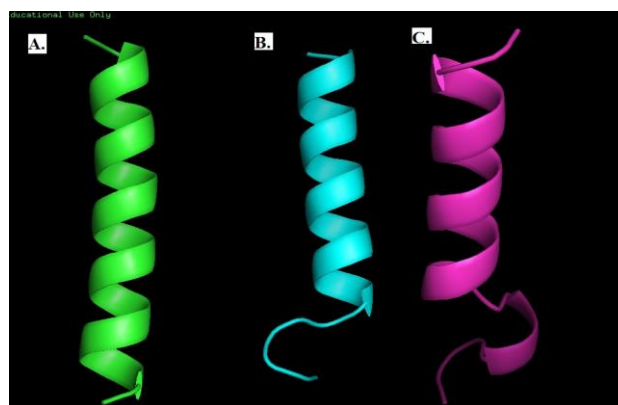

Figure 3: The prediction of secondary structure ofS6 peptide and its analogsusing software Pymol.
A. S6 (Green), B. S6M (Blue) and C. S6SCR (Magenta). By altering the more hydrophobic amino acids with less hydrophobic one changes the %age helicity of the three peptides which is quite evident from the predicted structure.
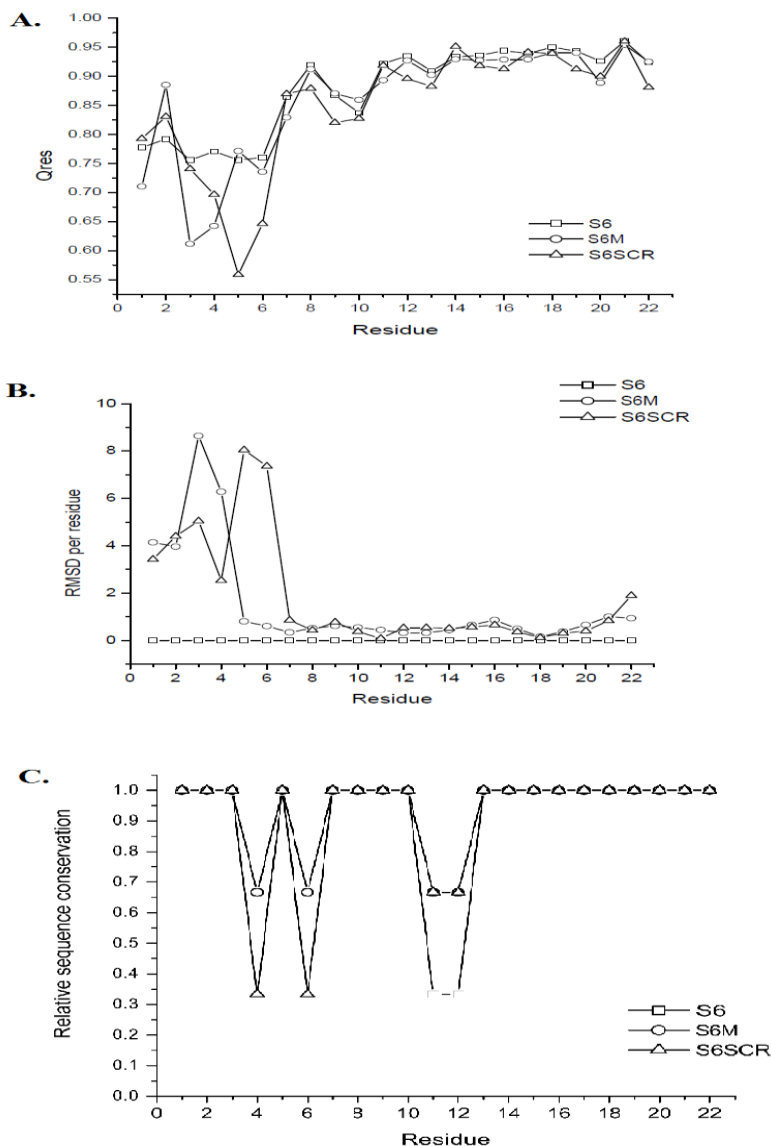
Figure 4: Comparison of structure of S6 peptide and its analogs;
The pdb files shown in figure 3 were used to align three sequences and do further analysis in VMD software using muliseq command prompt **A.**graph forQ_{res}*versus* residue number, **B.** RMSD per residue *versus* residue number taking S6 as standard molecule and C. relative conservation score of S6, S6M and S6SCR.

Various domains in the ion channels assemble themselves in the form of tetramers (Blunck & Batulan, 2012; Labro & Snyders, 2012). For such assembly, the domains should have a proper shape and secondary structure so that they may fit into each other in a proper manner. From this analysis we have shown that the amino acids in the S6 domain are arranged in such a manner that they provide the ion channel a proper shape so that they may assemble in the membrane properly and support the ion conduction as in case of S6. If more hydrophobic amino acid isoleucine is interchange with less hydrophobic glycine followed by alanine and isoleucine in S6SCR it proves to be fatal for the channel formation. It also reduces the helicity of peptide from 19 to 14%. These mutations cause the 2nd Threonine to fall in the outlier region which could cause the non-functioning of the channel or conduction of ions at higher voltages. Whereas by only one mutation at the center of the channel (isoleucine with glycine) in the S6M makes higher helicity (34%) and channel formation does takes place but it introduces bad angles in the Ramachandran plot hence its behavior also becomes abnormal.

CONCLUSION

We have explored the possible reasons for functioning of ion-channel by changing the amino acids in S6 domain of KvAP, potassium channel. We found that mutation in the pore domain especially more hydrophobic isoleucine with less hydrophobic alanine causes the abnormal functioning of the channel. Secondly, Glycine could also affection channel functioning as in case of S6M. These mutations cause the change of helicity of the peptides. At the end, it is eluded that the ion channel function is dependent on the helicity of the peptide. A proper helicity of the peptide is required for normal functioning of the peptides which is dependent on the arrangement of amino acids within each domain. This is the reason why evolution has conserved certain amino acids that are critical for ion channel functioning.

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