Indian Journal of Biochemistry & Biophysics
Vol. 63, June 2026, pp. 700-710
DOI: 10.56042/ijbb.v63i6.9407

Conformational and docking analyses of the frenatin 3 peptide, an inhibitor of nNOS enzyme and a ligand for Ca^{2+}-calmodulin

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Received 08 April 2024; *revised 29 April 2026*

The bioactive peptides are one of the promising drugs to curb a number of diseases. Recently, eighty peptide based drugs have been approved by Food and Drug Administration. The skin peptides of frogs have been studied for their activities in large number of cellular and biomolecular processes *i.e.*, up regulation, down regulation and inhibition. The frenatin 3 peptide, from the skin of *Litoria infrafrenata*, has been studied for conformation and secondary structure elements. The simulation studies have been carried out for frenatin 3 peptide and its computational mutants. The results suggested the most populated secondary structural elements, their stability and also reflect the effect of mutations on structure. Further, the docking studies with known targets of frenatin 3 were revealed the peptide-protein interactions i.e., backbone-backbone, side chain – side chain and backbone – side chain. The hydrophobic core formed by EF hand motif of calmodulin plays very important role in peptide-calmodulin interactions. The inhibition of Ca^{2+}-calmodulin complex by frenatin 3 peptide, consequently the inhibition of nNOS synthase, predicted to occur from molecular docking studies. The peptide-protein hydrophobic interactions considered to be the main player in this case. The homology and immunoinformatic studies on these peptides were performed to test their immunological role and thus proposed for further experimental studies.

Keywords: Ca^{2+}-Calmodulin complex, Frenatin 3, MD simulations, nNOS enzyme, Peptide drugs

The intriguing aspect of frog skin peptides is their multifaceted activity *i.e.* antimicrobial, anticancer, antiviral activity, participation in immunological responses and anticoagulation process. The few peptides from genus *Litoria* form complex with Ca^{2+}-calmodulin protein. The latter is a cytoplasmic protein, regulates or affects a wide range of cellular functions. The Ca^{2+}-calmodulin complex interacts with Ca^{2+}/calmodulin domain of nitric oxide synthase (nNOS) and activates the enzyme[1]. The natural amphipathic peptides have been shown to bind Ca^{2+}-calmodulin very tightly with $K_{diss} > 10^{-7}$ M^2 . The peptide frenatin 3 (2181.62 Da) from skin secretion of frog *Litoria infrafrenata* found to inhibit function of calcineurin, regulated by Ca^{2+}-calmodulin complex, which activates nNOS enzyme[3,4].

The amino acid sequence analysis revealed intrinsic amphipathic character of frenatin 3 peptide *viz.* Gly (22.7%), Leu (18.2%), Val (13.6%) and Lys (9.1%). The C-terminal of peptide has free *–COOH* group like the other frog host-defence peptides. The GX_3G motif (where X can be any amino acid residue) is consecutively repeated twice like bacteriocins, bombinins, plasticins[3]. The conserved GX_3G motif is also present in the membrane as well as in soluble proteins in which these motifs are engaged in helix to helix interactions[5], but nature of the helices remains debatable. Generally, amino acid residues Ala, Glu, Leu and His are present in the inner helical cores of proteins[6]. The polar residues (His, Lys and Arg) at the C-terminal ends are found preferentially at the helix-coil boundary regions in the proteins. The conserved GX_3G motif was observed first time in Glycophorin A[7] and since then it has reported in water-soluble[5] as well as membrane proteins. The motif is important for homodimerization. Glycine residues provide a shallow groove for the second helix[8,9]. The transmembrane interacting peptides may be useful for therapeutic development as they can interact with TM (transmembrane) regions of the receptors. GPCR receptors are target for only 30% of therapeutic drugs under study. The number of orphan GPCRs is increasing with more research on membrane proteins[10].

Experimental studies have shown that frenatin 3 adopts a helical conformation in membrane-mimicking environments, suggesting that its structure is highly sensitive to environmental conditions. The

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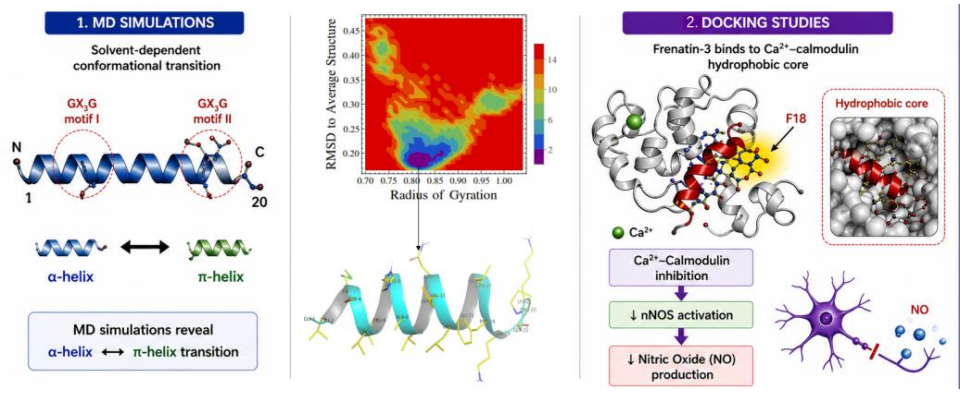
Suppl. data available on respective page of NOPR



Graphical abstract

2D NMR spectroscopic results for frenatin 3 showed that first 14 residues exhibit a helix in membrane mimicking solvent TFE/water. The peptide shown the persistence of helical character at the N-terminal in water solvent[3]. Thus, it was worth to explore the conformational behaviour of frenatin 3 peptide in different solvents. The molecular dynamics (MD) simulations throw light on the type of secondary structure favoured by GX_3G motifs and role of -OH group at the C- terminal.

The present work aims to (i) explore the conformational dynamics of frenatin 3, (ii) examine the structural role of conserved residues through systematic mutations, and (iii) investigate its interaction with Ca2+-calmodulin to better understand its inhibitory mechanism, iv) the molecular interactions of frenatin 3 with Ca^{2+}-calmodulin protein complex. The theraputic potential of the frenatin 3 peptide was proposed in relation to calmodulin and its role in other diseases/metabolic processes. The conformational behaviour of frenatin 3 has also been investigated by increasing charge on the peptide by computational mutation of Ser and His with Lys residue *i.e.* S4K, H8K mutant (Table 1). The homology between human proteome and frenatin 3 has been investigated.

Materials and Methods

Starting geometry

The 2D-NMR spectroscopic results suggested the alpha-helix as main secondary structure adopted by the peptide frenatin 3 in TFE and water. Therefore, the alpha-helical structure with φ = -57°, ψ = -47° and ω = 180° was generated using PyMOL[11]. The positive charge on native frenatin 3 peptide has been increased by computational mutations at Ser4 and His8 with Lys residues in the frenatin 3 peptide. Further simulations on peptide systems were also performed by mutating glycine residues with alanine residues systematically, (Table 1).

Table 1 — Peptides studied in water and DMSO

S. No.	Peptide	Mutation
1	GLMSVLGHAVGNVLGGLFKPKS-OH	Native
2	GLMKVLGKAVGNVLGGLFKPKS-OH	S4K, H8K (Lys rich)
3	GLMSVLAHAVGNVLGGLFKPKS-OH	G7A
4	GLMSVLAHAVANVLGGLFKPKS-OH	G7A, G11A
5	GLMSVLAHAVANVLGALFKPKS-OH	G7A, G11A, G16A
6	GLMSVLAHAVANVLAALFKPKS-OH	G7A, G11A, G15A, G16A
7	GLMSVLAHAVANVLAALFKAKS-OH	7G/A, G11A, 15G/A, 16G/A, 20P/A

All glycine residues and proline-20 replaced with alanine. The mutated peptides have been investigated to understand the structural and functional role of these residues in the peptide.

MD simulations

All the generated geometries of peptides were studied with GROMACS package (version 5.1.4) using GROMOS96 G43a1 force field[12,13]. The N-terminal was protonated ($^{+}NH_3$) and C-terminal Ser was changed for free -CO_2H group using PyMOL software. All atoms of the systems were considered in an explicit manner. The peptide was placed in centre of cubic box at a distance of 1.0 nm from edge of the box. The starting structure for each peptide was soaked in water SPC (single point charge) model and simulated using periodic boundary conditions[14]. The positive charges on the systems were neutralized by adding chloride ions. Energy minimization, with the steepest descent method, was done to remove the steric clashes between the atoms of the systems. The equilibration of the systems was done first under NVT conditions for 100 ps followed by NPT conditions for

100 ps using modified Berendsen thermostat for temperature and pressure coupling protocol. The long-range interactions were calculated using particle mesh ewald method. The van der Waals interaction cut off was kept 1.0 nm to calculate short range interactions[15]. LINCS algorithm was used to constrain the bond lengths[16]. Final production run for 10 ns were performed for frenatin 3 peptide and its mutants to see conformational change as function of time in explicit SPC water model.

Production runs of 10 ns were carried out for each system. Multiple independent simulations were performed to ensure reproducibility. While longer simulations can provide extended sampling, short-timescale simulations are widely accepted for evaluating structural stability and dominant conformational states in small peptides.

Analysis of MD simulations

Trajectory analyses included RMSD, RMSF, radius of gyration, solvent-accessible surface area, hydrogen bonding and secondary structure determination using DSSP. Free energy landscapes (FEL) were constructed to identify energetically favourable conformations. The trajectories obtained from MD simulations were analysed to see conformational behaviour of frenatin 3 and its computational mutants. The analysis of the trajectories was performed for RMSD, RMSF, radius of gyration (Rg), solvent accessible surface area and secondary structure. Hydrogen bonds were calculated for intra-molecular and solvent-peptide during simulations. For CO--NH hydrogen bond default values for donor-acceptor distance <0.35 nm were considered[17]. The principal component analysis, free energy landscape and cluster analysis were explored from independent multiple MD simulation trajectories for each peptide. The analysis of the results has been performed by Gromacs tools *viz*. *gmx_trjconv, gmx_enegry, gmx_rms, gmx_gyrate, gmx_rmsf, gmx_sasa, gmx_chi etc*.

Docking studies

The molecular docking was performed with AutoDock vina version 4.2[18]. The coordinates for frenatin 3 peptide in α-helix model were generated by PyMol. The structure for calmodulin was retrieved from RCSB protein data bank (PDB ID: 1CLL) and the solvent molecules were removed. The grid dimensions were set to 30 Å × 28 Å × 28 Å with grid centre defined at 13.872, 25.949,12.515 for x, y, and z dimensions, respectively. The docking results were analysed using PyMOL[11]. Further, the docking studies were performed using available online tools for protein-peptide docking[19-23]. Protein–peptide docking was performed using AutoDock Vina and validated using multiple docking platforms. The docked complexes were further refined using MD simulations to assess stability and interaction persistence. The analysis of simulation was done with PyMol and the graphics were prepared to analyse the peptide-protein interactions.

Homology Search using NCBI BLASTp server:

A sequence similarity search for frenatin-3 was performed using NCBI BLASTp against the *Homo sapiens* reference proteome. The search returned multiple hits; however, these were confined to short alignment regions and associated with relatively high E-values.

Results and Discussion

Conformational stability and simulation reliability

The RMSD profiles showed rapid convergence and stable plateau behaviour, indicating that the peptide systems reached equilibrium within the simulation time frame. The radius of gyration remained consistent, confirming structural compactness. Free energy landscapes further revealed well-defined minima, supporting the presence of stable conformational states. Together, these observations demonstrate that the simulations capture the dominant structural ensemble of the peptide despite the moderate simulation length.

The fluctuations were observed in both GX_3G motifs of frentain 3 shown in (Suppl. Fig. 1). Therefore, the G→A, mutations were employed computationally followed by MD simulations in water and DMSO as solvents. The role of proline residue present at C-terminal end of the membrane interacting peptides was advocated elsewhere[24-26]. Therefore, the computationally mutated P20A frenatin 3 peptide was considered for MD simulations to see the role of proline-20 residue at the C-terminal.

The conformational preferences of frenatin 3 and its mutants peptides were observed as a function of simulation time and the results shown in (Suppl. Fig. 1a) for the rmsd values (root-mean-square deviation values in nm) for the native frenatin 3 and its Lys rich computational mutant. The stability of MD simulations have been checked after plotting average Cα RMSD values. It showed hardly any

deviation at Cα positions which supported the stability of peptide backbone. Thus, there were the least changes in secondary structure of peptide during course of simulations. The Lys rich peptide showed fewer deviations in the Cα atoms as a function of time and the stability was enhanced due to G→A mutation at position 3, 7, 15, and 16 in other mutants. It is due to the highest propensity of alanine residue to form α-helix[27]. In native frenatin 3 peptide, the glycine residues showed more deviations at Cα atoms as it lacks the true side chain i.e., only a proton is present as a side chain group. The root-mean-square fluctuations for the Cα residues were analysed and compared with Lys rich mutated peptide, (Suppl. Fig. 2). In terms of free energy Δ (ΔG), glycine is ~1 kcal/mol less favourable with respect to alanine for adoption of helical character[27]. Therefore, to reduce fluctuations in vicinity of Gly residues, G→A, computational mutations were carried out in native frenatin 3 peptide and their effect on secondary structure was investigated.

Secondary structure and solvent effects

The peptide exhibited distinct conformational preferences depending on the solvent environment. In aqueous conditions, π-helical structures were frequently observed, whereas low dielectric conditions favoured α-helical conformations. This behaviour is consistent with experimental findings and highlights the adaptability of the peptide structure.

The DSSP analysis for frenatin 3 revealed that the N-terminal contains more helical content than C-terminal, the latter has turns or bends as secondary structural elements. The middle residues mostly adopted π-helical structure shown purple (Fig. 1). The representative structure from the free energy calculations and stride analysis further showed preponderance of π-helix in mid-region of frenatin 3 peptide and its mutants except G7A and Lys rich peptide. The residues of mutant peptides have been populated more in helical structure than the native peptide in both the solvents, (Suppl. Table 1 and Suppl. Figs. 3-5), in water and DMSO solvents, respectively. The other DSSP plots for the peptides with G→A, mutations are shown, (Suppl. Fig. 3). The secondary structure elements in these peptides revealed that the mutants G→A (7, 11, 15, 16 and 16) and P20A favoured α-helical character towards N-terminal while C-terminal populated with π-helix. Further, the α- and π-helical characters are interchangeable during course of MD simulations[28].

Glycine residue have the smallest side chain and hence can adopt φ, ψ values in any of the four regions of Ramachandran map. Glycine residues have been observed to break α-helix or found to have low propensity for α-helix. The GX_3G motifs permitted close packing of dimers and enhance helix-helix stability along with increased van der Waals contacts, this increases long-range electrostatic interactions between helices. Further, the potential for Cα-H hydrogen bonding. The G→A mutation stabilized the helical content in all studied mutant peptides. Furthermore, the side chain of Lys residue has hydrophilic ε-amino group. The hydrophilic amino group in Lys rich peptide increases overall charge on the peptide mutant in addition to hydrophilic character. Thus, the peptide S4K H8K, adopts β-sheet character after simulations.

Free Energy Landscape (FEL)

The conformational space for the peptide has been calculated from multiple simulated trajectories for free energy calculations. The free energy landscapes predicted the conformation with the lowest Gibbs free

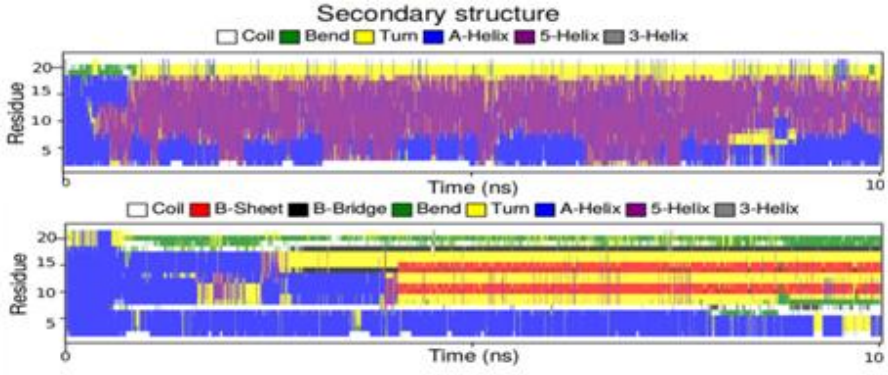


Fig. 1 — Standardized secondary structure assignment (DSSP) analysis for native frenatin 3 and S4K, H8K mutant peptide predicted the π-helix in the former and β-sheet & turn in the latter

energy (ΔG). The average conformations obtained from FEL the lowest energy basins for the frenatin 3 and mutant peptide shown in (Fig. 2). The analysis of average or representative conformation with stride server, suggest that the π-helix is the most populated secondary structure in native frenatin 3 peptide. The GX_3P motif is present at C-terminal of native frenatin 3 peptide. The functions of GX_3P motif has been reported in chloride ion transporter[29]. Further, proline plays as a helix breaker in water-soluble peptides/ proteins. It is clear from the DSSP plot as well as FEL conformation, the helix is absent at the C-terminus of native frenatin 3 peptide. In case of AMPs, it is a conserved residue that helps for partial insertion in to the cell membrane[30,24]. Hence, the role of Pro-20 residue in the native peptide was studied by Ala scanning (*i.e.*, P20A mutation) and simulation results showed preponderance of helix in this region (Suppl. Fig. 3).

Helix stability as a function of simulation time

α-helix was the starting conformation for simulations both in water and DMSO solvents. It has been observed that the φ, ψ values of most of the residues remained in helical region except a few. The helix radius was found to lie between 0.23–0.27 nm between α-helix to π -helix transitions as shown in (Suppl. Fig. 2). The helix stability in terms of intra-protein hydrogen bonds is found to be less in Lys rich mutant peptide than native frenatin 3. The intra-protein hydrogen bonds disappear in Lys rich peptides. It occurred as the positively charged side chains interacted with water. Further, the charged amino acid residues improve the solubility but reduce the helix stability of proteins[31]. The structure of biomolecules is directly influenced by solvation, solvent molecules or hydration shells. The structure of peptides changes with different solvents and number of solvent molecules surrounding the protein/peptide[32,33]. The cationic peptides have been shown to interact better with negatively charged cancer cell membranes[34]. The ΔG of solvation is observed more and solvent accessible surface area (*sasa*) less in case of Lys rich mutant in comparison to frenatin 3 peptide. The interactions like protein-water and intra-protein hydrogen bonding contributes in stability of the structure as shown by plots in (Fig. 3). The analysis of plots revealed that there is an increase in area (nm^2) of 4, 5, 8 and 17th residues and decrease in area of 7, 11 and 15th residue as expected due to G→A, mutations.

Effect of mutations on structure

The amino acid sequence determines three-dimensional structure of proteins and peptides. A mutation in the sequence influence structure as well as function[35-37]. The frenatin 3 peptide was computationally mutated at various sites to study the effect and importance of residues present in native frenatin 3 peptide. The analysis of simulation for backbone dihedral angles revealed a change in the region for H8K mutation (inset in Figure 4b). The φ, ψ distribution Figure 4b showed the His8 residue fall in helical region. The Lys8 residue adopted φ, ψ

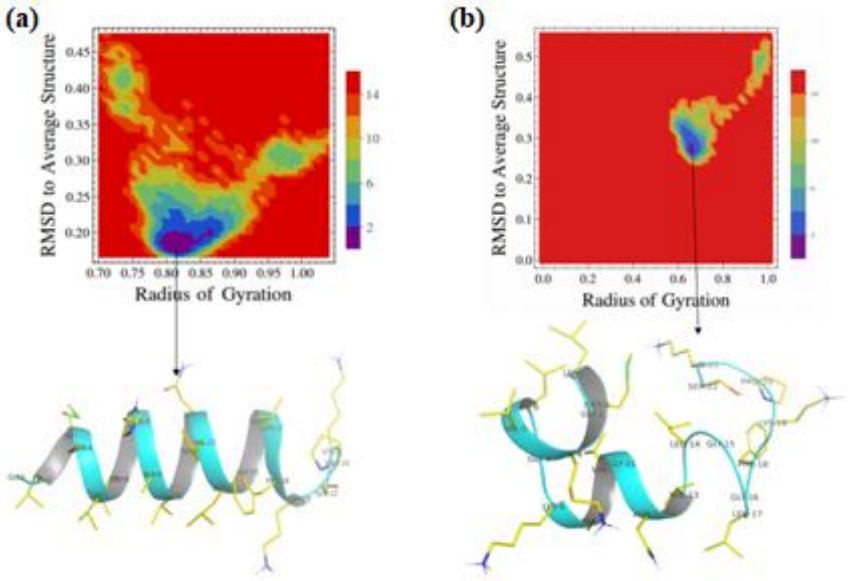


Fig. 2 — Free Energy Landscape of the trajectory and representative conformations of (a) frenatin 3; and (b) S4K, H8K mutant after simulation in explicit water solvent

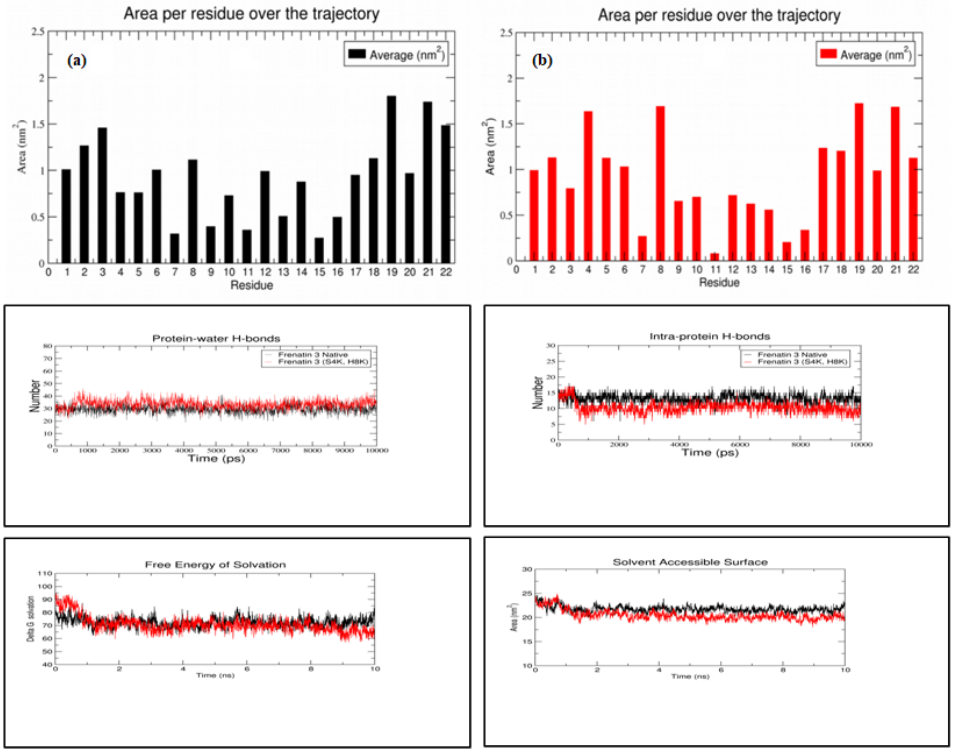


Fig. 3 — The plot for area per residue for native frenatin 3 and Lys rich mutated peptide (S4K, H8K). The plots for hydrogen bonds intra-peptide and peptide-water (upper panel) and free energy of solvation, solvent accessible surface area for both native and Lys rich mutant

angles belong to extended and helical region in Ramachandran map. The S4K mutation favoured more φ, ψ angles in α-helical region of the map (Fig. 4a) although, the quadrant remained same.

Similarly, the φ, ψ distributions for glycine and alanine residues shown in (Fig. 5 c-f). The plots clearly depicted that the G→A mutation results in order-ness in peptide backbone, α-helical region of third quadrant. It is evident from the φ, ψ distributions, the residues Gly15 and Gly16 (*black dots* in 5 e & f) found in 2nd and 3rd quadrants. The alanine mutations favoured the φ, ψ values only in 3rd quadrant (α-helical region). The φ, ψ distribution for P20A residue mutation depicted similar behaviour. The adoption of φ, ψ values in α-helical region is due to high propensity of alanine to form an α-helix[38].

Systematic mutations revealed that glycine residues contribute to flexibility, while alanine substitutions enhance helical stability. Charge-enhancing mutations increased solubility but reduced structural rigidity. The P20A mutation confirmed the role of proline as a helix disruptor. These results demonstrate that targeted mutations can effectively modulate peptide structure and stability.

Amide bond analysis from simulated trajectories

The deviation in ω dihedral angle was reported to be ±25° by computational as well as experimental methods. Bour and coworkers have explored the non-planarity of peptide bond and further reports suggested the deviations in amide bond near ± 25° in peptides[39,40].

The analysis for ω dihedral distributions are pretty clear that all amide bonds remained in an energetically favoured *trans* configuration, (Fig. 5). The deviations in the amide bond (CO--NH) were plotted and it was observed that all ω angles restricted to the region ~ 180° ± 20° except Pro20 residue. The ω dihedral angle for Pro20 residue, shows a slightly different behaviour in frenatin 3 native peptide was found because the values were near ~150°, (Fig. 5a).

The Δω values for the representative structures for S4K, H8K as well as other mutants were plotted in comparison to Δω angles in energetically most populated conformation of native and S4K, H8K forms of frenatin 3 peptide. The changes are less in the middle and reduced at residue positions 2-4 and 16 significantly, while increased 12-14 and 21 in S4K, H8K mutant as compare to the native frenatin 3 peptide.

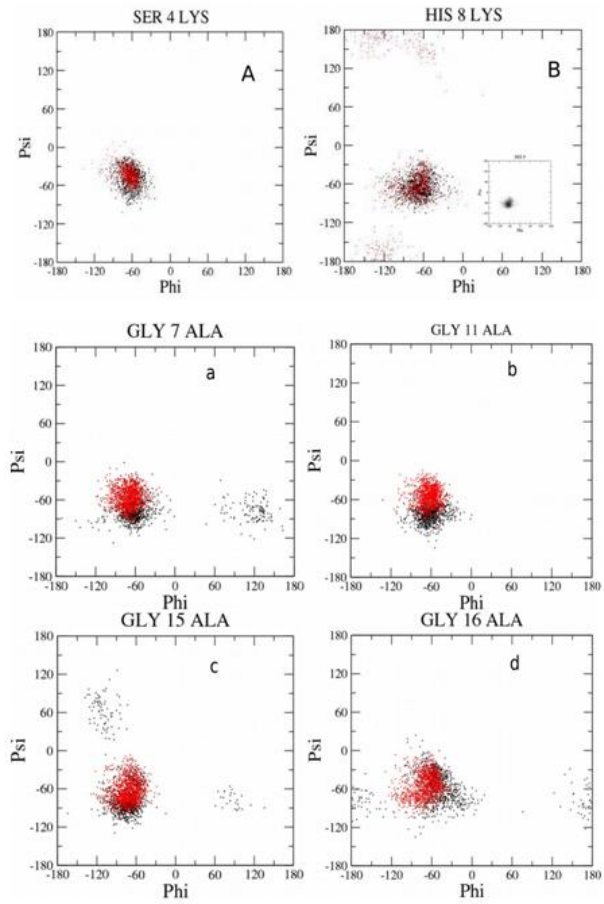


Fig. 4 — The φ, ψ distributions for S4, H8 (*black dots*) and L4, L8 (*red dots*) of frenatin 3 native and mutant peptide, respectively for last 5 ns MD simulation trajectory. The inset plot shows the region occupied by His8 in native peptide. The φ, ψ distributions for Gly to Ala mutated residues (black dots for Gly and red for Ala). The distributions show that the G→A mutation restricted φ, ψ angles in helical region of Ramachandran map

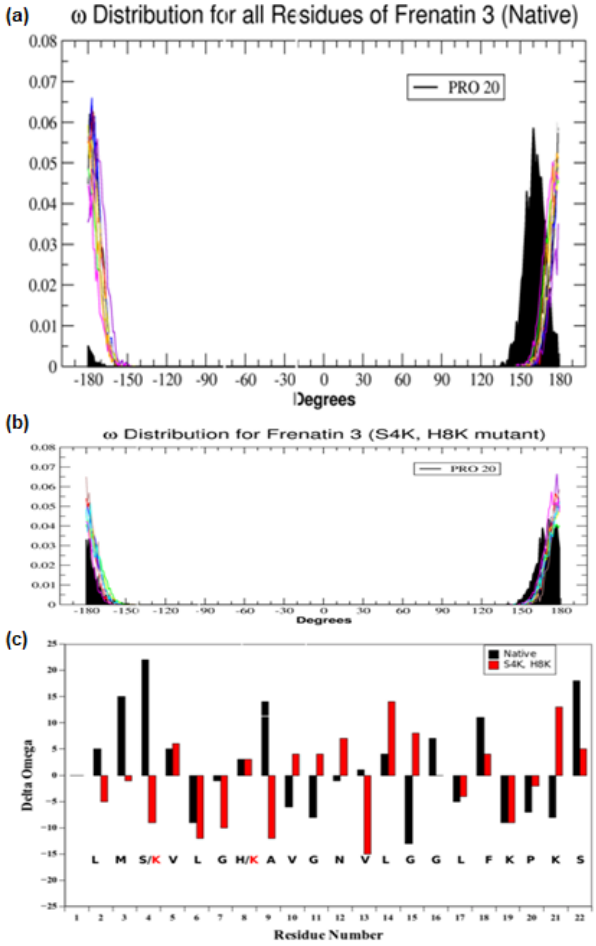


Fig. 5 — The bar plot for the change in amide dihedrals (Δω) from most stable conformations of frenatin 3 native and (a) S4K, H8K mutant; (b) after MD simulations in water; The mutations shown red; (c) The amide bond, ω dihedral distributions for (i) native frenatin 3 and (ii) S4K, H8K mutant peptide after simulation studies in water. The amide bond dihedral in both cases showed different distribution than other residues

Cluster analysis

The cluster analysis from the simulated trajectories revealed 10 and 17 populated conformations for native and S4K, H8K mutant, respectively. The largest group for native peptide found to acquire helical structure, starts from 3rd residue and ends at 17th residue. The φ, ψ values thrown light on length and type of helical region. The S4K, H8K mutant has a small α-helical segment at the N-terminal and rest is an extended helix or unordered secondary structure. Further, the microstates like 4, 5, 7 and 10 has more helical content, even the fourth microstate has a long helical structure (Fig. 6).

Comparison of representative structure of Frenatin 3 with its studied mutants

The representative structures obtained from trajectory analysis were compared for φ, ψ angles for each residue of the peptides (Suppl. Fig. 8). Terminal residues were discarded, since their (ϕ, ψ) angles are not defined. The plot analysis clearly depicted the residues L^{14}, F^{18}, P^{20} found extended region, G^{11} in turn while K^{21} (left-handed α-helix) and G^{7} fall in right-hand side of 1st quadrant of Ramachandran map for S4K, H8K mutant. Similarly, the comparison of the φ, ψ angles adopted by the other mutants are compared with the native peptide, data not shown. Here, a point must be emphasized that the G→A mutation directs the residues to fall in helical region, as alanine residues have the highest propensity to form right-handed α-helix.

Docking results

The N- and C- domains of EF hand motif present in Ca^{+2}-calmodulin protein are the energetically most

favoured regions for native frenatin 3 peptide. It is predicted from different molecular docking tools used in the study and the N-terminal sequence (1-8 residues) of native frenatin 3 are found to interact

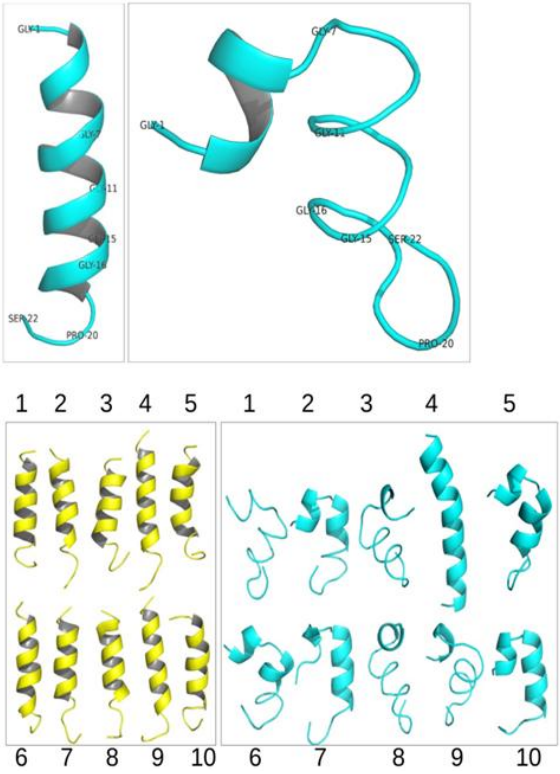


Fig. 6 — The cartoon displays for native frenatin 3 (left) and S4K, H8K mutant (right) showed the helix preference for native and only small helix is present at the N-terminal of S4K, H8K mutant. The most populated clusters showed for frenatin 3 native (yellow) and S4K, H8K mutant (cyan)

with the helices present in N and C domains, as shown in (Fig. 7). Similar results have been predicted from other online tools as shown in (Suppl. Fig. 6). The trajectory analysis of the docked complex of native frenatin 3 -calmodulin revealed that F^{18} residue remain in the hydrophobic core made by the helices belongs to C terminal EF hand motif of calmodulin protein (Fig. 7 e & f).

Although, initial docking was performed using static methods, subsequent molecular dynamics simulations allowed relaxation and refinement of the complexes. Consistent binding modes across multiple docking platforms further support the reliability of the predicted interactions. Detailed analysis of the docked complexes revealed that frenatin 3 binds within the hydrophobic pocket of the EF-hand domain of Ca2+-calmodulin. Hydrophobic interactions dominate the binding, particularly involving F^{18}, which is deeply embedded in the protein core. Additional stabilization arises from hydrogen bonds and electrostatic interactions. The peptide aligns along the helical domains of calmodulin, potentially restricting its conformational flexibility and interfering with nNOS activation.

Frenatin 3 possesses features desirable for therapeutic peptides, including small size, amphipathicity, and structural adaptability. Its interaction with Ca2+-calmodulin suggests potential for modulating signaling pathways.

The interaction analysis indicates that the binding of frenatin 3 to calmodulin is largely governed by hydrophobic forces, with F^{18} playing a central role in

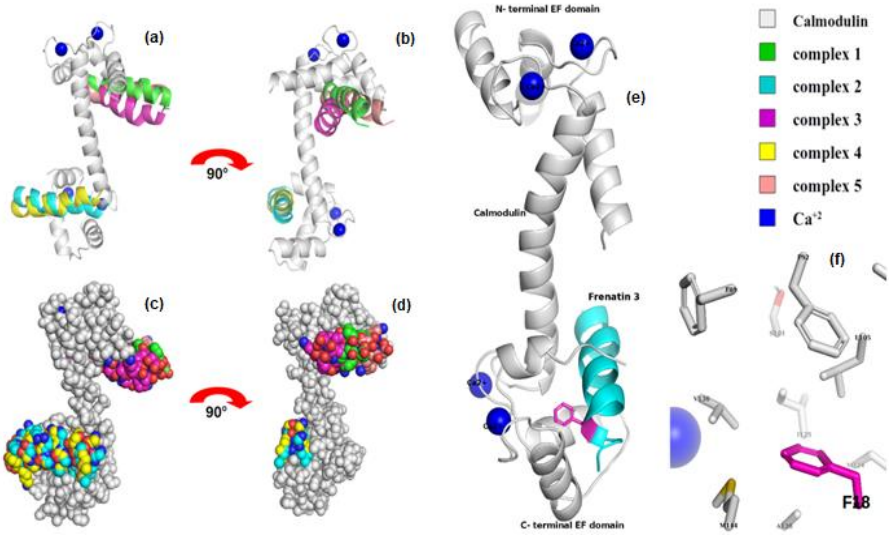


Fig. 7 — The docking results for Ca^{+2}-calmodulin with native frenatin 3 peptide (a) the cartoon representation of the first five results; and (b) the rotated view by 90 degrees, of the same

stabilizing the complex. Additional contributions arise from van der Waals interactions, along with potential involvement of π–π and cation–π contacts. In contrast, hydrogen bonds and other polar interactions are relatively sparse, suggesting that hydrophobic packing serves as the primary driving force for the formation and stability of the complex (Suppl. Fig. 9).

Homology search against human proteins

A sequence similarity search for frenatin-3 native was performed using NCBI BLASTp against the *Homo sapiens* reference proteome. Owing to the short length of the peptide, search parameters were adjusted to enhance sensitivity. No significant full-length or near full-length alignments were detected ($E < 0.001$). These findings suggest that frenatin-3 does not share meaningful sequence similarity with human proteins, supporting its potential specificity and a low likelihood of off-target interactions.

Physicochemical properties

The peptide frenatin 3 was analysed for its physicochemical, safety and functional properties using ProtParam and complementary computational approaches. It comprises 22 a.a. with a MW of 2181.62 Da and a theoretical pI of 10.00, indicating a basic nature due to the presence of lysine residues and absence of acidic amino acids. The instability index (17.15) classifies the peptide as stable, while a high aliphatic index (115.00) suggests good thermal stability. A positive GRAVY value (0.664) reflects its hydrophobic character, supported by enrichment of glycine, leucine and valine residues, which together contribute to structural flexibility and stability. The absence of aromatic residues implies negligible absorbance at 280 nm.

Safety assessment indicated that the peptide is non-toxic, as predicted by ToxinPred3 and supported by HyPeptox-Fuse tool. Structural modelling using PEP-FOLD3 suggested partially ordered conformations with α-helical tendencies, consistent with peptides capable of membrane or protein interactions.

Preliminary immunoinformatics predictions were also performed for MHC-I and MHC-II, IFN-gamma. The peptide is a good inducer of Ifn-gamma, therefore it may stimulate $CD4^+$ Th1 cells.

Conclusion

The present study throws light on the conformation of frenatin 3 peptide in different solvents and revealed the environmental dependent behaviour of the peptide. The solution NMR structure of frenatin 3 in water and TFE, a membrane mimicking solvent, was found to be an α-helix[3]. The previous studies showed that the secondary structure of peptides depends upon its environment[41]. The present molecular dynamics study in different solvents implied that the native peptide adopted a π-helical structure in water while an α-helix is the major-populated secondary structure, in the representative conformation obtained from free energy calculations. The S4K, H8K mutant resulted in an opened helix or unordered conformation in water, the π-helix found in middle of peptide in DMSO solvent. The studied G→A and P→A mutants of frenatin 3 peptide preferred to acquire π-helical conformation in both solvents during the course of MD simulations. Interestingly, the functionally important feature of peptides interacting with membrane proteins, *the bulge* formed by the GX_3G motif disappeared after G/A mutation in studied systems. Hence, this study showed the solvent dependent behaviour of frenatin 3 peptide, which is a necessity for the peptide to function. The conformation of frenatin 3 in water was more flexible at the glycine revealed by NMR spectroscopic data and less flexible in TFE/water solvent[3]. Therefore, the simulation study is in correlation with the available experimental results. Further, the study suggested that frenatin 3 peptide has environment dependent conformational behaviour. The docking studies support the binding of Ca^{+2}-calmodulin to helical peptides and thus frenatin 3. The peptide might inhibit the cytosolic proteins like Ca^{2+}-Calmodulin complex.

However, further experimental studies are required to evaluate pharmacokinetics, toxicity and delivery mechanisms before therapeutic applications can be realized. Preliminary homology analysis suggests low similarity with human proteins, indicating a reduced risk of cross-reactivity. Nonetheless, detailed experimental immunological studies are necessary to confirm safety. Simulations in a low dielectric environment demonstrate that the peptide adopts conformations consistent with membrane-active peptides. While explicit membrane simulations were not performed, the results provide insight into environment-dependent structural behaviour. Therefore, frenatin 3 is a stable, hydrophobic and biologically compatible peptide with a favourable safety profile and promising interaction potential, warranting further experimental validation.

This study provides a comprehensive analysis of the conformational dynamics and interaction mechanisms of frenatin 3. The peptide exhibits strong environment-dependent structural adaptability and stable binding to Ca^{2+}-calmodulin. The findings support its potential role as an inhibitor of nNOS-related pathways and highlight the importance of sequence modifications in tuning peptide structure and function. Future work involving extended simulations, membrane models and experimental validation will further clarify its therapeutic potential.

Acknowledgement

We are thankful to Shama for proofreading the manuscript. This work is dedicated to late Prof. FS Nandel, who had a big devotion for peptide structure and drug design. I am indebted to Professor FS Nandel for his suggestions in writing this manuscript.

Conflict of interest

The author declares no conflict of interest

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