Understanding the Structural and Dynamic Effects of Protein Palmitoylation: A Computational Approach

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Abstract
Calnexin, a critical chaperone protein in the endoplasmic reticulum (ER), plays a vital role in glycoprotein folding and quality control. This study investigates how palmitoylation, a post-translational lipid modification, affects calnexin's structural and functional dynamics. Using molecular dynamics (MD) simulations, we analysed the impact of palmitoylation at specific cysteine residues on the protein's stability and interactions. Both palmitoylated and non-palmitoylated forms of calnexin were examined to better understand its role in ER quality control and the glycoprotein folding process.

Our findings revealed that palmitoylation induces conformational changes in calnexin, improving its stability and potentially altering its interactions with ER-associated proteins. These structural changes suggest that palmitoylation plays a significant role in modulating calnexin's function, which may have broader implications for protein dynamics and cellular homeostasis. The results underscore the importance of lipid modifications in maintaining ER function and highlight potential links between calnexin palmitoylation and protein folding disorders.

Keywords: Calnexin, Molecular dynamics simulation, Principal Component Analysis.

Introduction

The endoplasmic reticulum (ER), encircled by a single lumen, indicates a continuous membranous network of tubular structures that participate in numerous processes including post-translational modification, protein synthesis, lipid, steroid production and Ca^{2+} storage.[6,9,14] On average, around one-third of the proteins expressed by human cells binds to ribosomes located on the ER where they are subsequently synthesized in different places inside their respective cells.[18]

Although the amino acid sequence determines a protein's native structure, molecular chaperones are often required for correct folding. One of the primary ways the ER promotes folding is through the N-linked glycoprotein pathway, regulated by calnexin and calreticulin.[13] Calnexin is an important membrane protein in the ER while calreticulin is present only within its lumen. Calnexin assists in quality control and protein folding, ensuring that only properly formed and folded proteins progress along the secretory pathway.[25] Unfolded or unassembled N-linked glycoproteins are retained in the ER while its interaction with ERp57[30] drives glycoprotein-specific disulfide bond formation. Calnexin also aids in folding MHC class I α-chains on the ER membrane. As they enter the ER, these chains are partially folded before calnexin binds to them.[8] Following this, calreticulin and ERp57 chaperone the MHC class I peptide-loading complex (PLC). β2-microglobulin has just bound to before tapasin takes over, connecting it to the transporter associated with antigen processing (TAP), thereby facilitating antigen presentation on the cell surface via MHC class I.

The mouse calnexin (Canx) gene, located on the negative strand of chromosome 11, has 14 exons. It translates into a 4281 bp transcript coding for a polypeptide made up of 591 amino acids. The human calnexin polypeptide is a type-I integral membrane protein weighing 67 kDa, although its mobility in SDS-PAGE can sometimes mislead it to be regarded as a 90 kDa substance due to its high acidity. The calnexin polypeptide consists of three topological domains: a transmembrane segment binding calnexin to the ER membrane, an N-terminal domain folded within the ER (the chaperone functional module) and a C-terminal region of 90 amino acids facing the cytosol, where many post-translational modifications occur.[8]

Earlier studies show that calnexin's N-terminal and C-terminal domains exhibit several low-affinity Ca2+ binding sites. However, the 3D structure of the luminal domain revealed a single Ca^{2+} binding site formed by Ser54, Asp416 and Asp97, a conserved location in both calnexin and calreticulin. Binding of Ca^{2+} to the ER luminal domain in calnexin leads to a conformational change. Additionally, the luminal domain binds ATP and Zn^{2+}, controlling conformational changes and enhancing anti-aggregation potential.[9] Recent studies have found that the topological domain undergoes post-translational modifications including palmitoylation[11], phosphorylation, sumoylation[8] and proteolytic cleavage[21].

Approximately 90% of calnexin molecules exhibit S-acylation at equilibrium, indicating continuous palmitoylation and depalmitoylation patterns.[13] Molecular dynamics simulations show that palmitoylation causes the topological domain to undergo several conformations relative to the transmembrane helix axis. Therefore, palmitoylation of the topological domains can affect

calnexin's ability to bind or interact preferentially with specific external proteins. Additionally, research indicates that calnexin's topological domain interacts with cytosolic proteins, influencing cellular functions. This suggests a vital role beyond being just a molecular chaperone by coordinating ER and cytosolic processes.

Moreover, these findings are keys in discovering and characterizing more functions of calnexin.[2] While numerous studies have explored the impact of palmitoylation in the transmembrane region, limited research has been conducted on its effects in the topological domains (luminal and cytoplasmic regions). This study aims to understand the impact of palmitoylation in these topological domains.

To better understand how palmitoylation affects calnexin (CNX), we used the non-palmitoylated CNX structure as a reference and introduced palmitoylation at the cysteine residue 195[24] within the luminal domain. It has garnered attention for its impact on calnexin's structure and function. Cysteine 195[24] is strategically located within the luminal domain, a region critical for calnexin's interactions with glycoproteins. Molecular dynamics (MD) simulations were conducted to observe the effects of this modification. Additionally, topological and essential dynamics analyses were performed to assess palmitoylation's impact on CNX function. The results highlight how palmitoylation influences CNX's role in overall function.

Material and Methods

Protein Preparation: The three-dimensional structure of calnexin was elucidated using the AlphaFold protein model with UniProt ID P35564 as in fig. 1(a). The non-palmitoylated form of calnexin in this structure served as the starting point for the MD simulations that we performed. Cysteine residue 195 was subjected to palmitoylation. This process involves transferring palmitoyl-CoA to the protein attaching it to cysteine residues via a thioester bond, resulting in a palmitic acid addition. The CHARMM36[22] force field and CHARMM-GUI 3.8[17] were used for incorporation of palmitate groups into the model as in fig. 1(b).

Molecular Dynamics simulation: The CHARMM-GUI[17] webserver was used in generating the simulating system. GROMACS[1] version 2024 was used to carry out the MD Simulations. The system was solvated with TIP3P water[28] before being ionized with 150 mM NaCl. The CHARMM36[22] force field was utilized to parameterize the atoms in the system. The simulated box was subjected to a periodic boundary condition and long-range electrostatic interactions were modelled using Particle Mesh Ewald (PME).[26] This system then underwent MD simulation with Gromacs 2024 consisting of two main stages: equilibration and production run.

The NPT ensemble was chosen for equilibration stage working at constant temperature and pressure equal to 310 K and 1 atm respectively where Langevin thermostat with the damping coefficient of 1/ps was employed along with Nosé-Hoover Langevin piston barostat having decay period equal to 25 fs. A distance cutoff for nonbonded contacts was set to be 12 Å while its switch distance was taken to be 10 Å. During production runs, an NPT ensemble was maintained over a duration of about 1000 ns rather than just utilizing longer simulation periods that could lead to excessive resource wastages.

Calnexin simulations were conducted in both forms (with or without palmitoylation) under similar conditions as the production run results utilized herein. We utilized GROMACS simulation package for analysing all data from simulation studies. The visualisation of structural figures was done using VMD[21] and PyMol[10] software programs. Xmgrace[23] tool created the graphs and plots.

Principal Component Analysis: For MD simulations to be able to determine the different protein conformations and major internal motions through which palmitoylation affects its global dynamics and conformational changes, the trajectories were analysed using principal component analysis (PCA)[3] or essential dynamics (ED). The correlated motions of protein atoms were extracted from various frames to determine how mobile it is. First, Cα atoms were used to create a variance/covariance matrix followed by diagonalizing that of covariance.

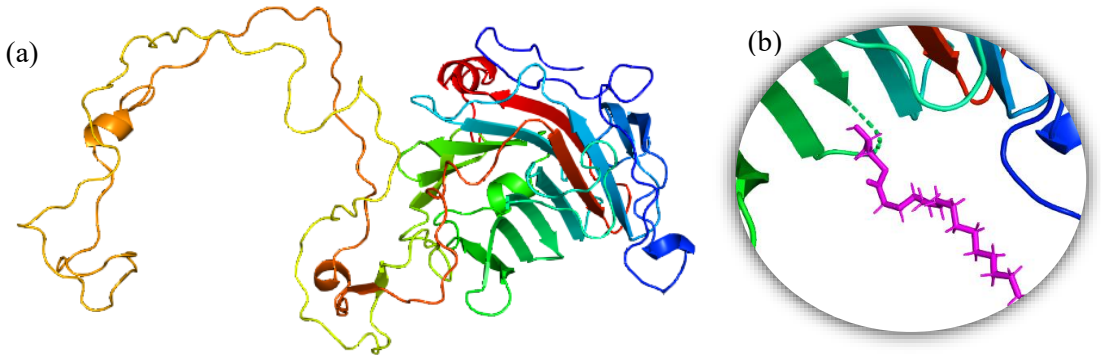

Figure 1: (a) The AlphaFold-predicted structure of mouse calnexin (UniProt ID: P35564) is shown, highlighting a lumenal domain spanning residues 21 to 482. (b) Enlarged image of attachment of palmitoyl tail at cysteine 195 position of Calnexin

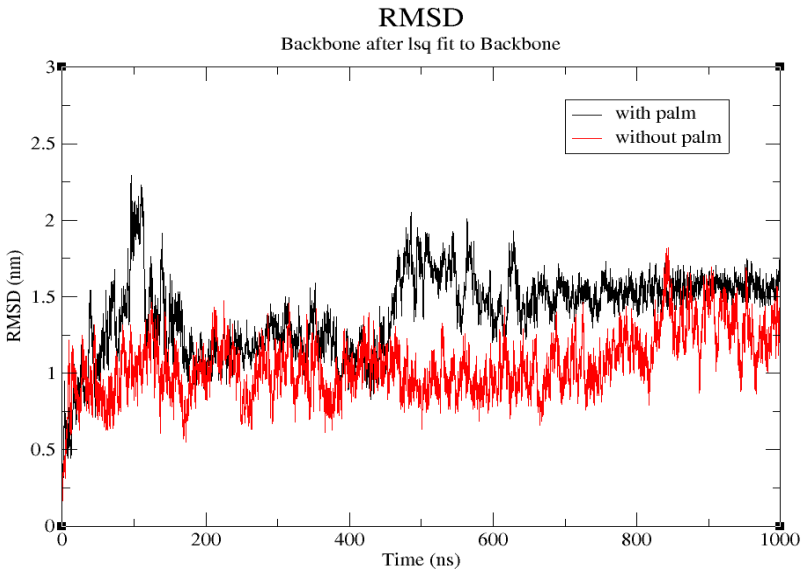

Figure 2: The backbone Root Mean Square Deviation (RMSD) of topological domain of Calnexin with and without palmitoylation

The translational and rotational motions were removed from atomic fluctuations and therefore, covariance matrix composed of protein movement directions established the first three N. The covariance matrix was diagonalized from the expression of linear transformation in Cartesian space as,

$$C = V\lambda VT$$

where λ represents respective Eigen vectors and Eigen values. The first two Eigen vectors (ev1 vs ev2) can be then projected on PCA using GROMACS to display the greatest movements. Using the Xmgrace tool[29], the results were examined and represented as 2D graphs.

Results and Discussion

Palmitoylation predicted to modify the structural topology of Calnexin: We evaluated the root mean square deviations (RMSD) of our simulations to determine structural stability, concentrating on changes in palmitoylated calnexin from its initial structure. The protein backbone alterations were depicted in figure 2. Previous studies have shown that proteins typically exhibit stable patterns of root mean square deviation (RMSD) in their alpha carbon (Cα)[12] atoms, providing insight into structural stability and conformational integrity.

In our molecular dynamics (MD) simulations, the non-palmitoylated form of calnexin displayed a fluctuating RMSD pattern, gradually stabilizing after approximately 200 nanoseconds (ns) of simulation time, followed by an additional 80 ns of minor deviations before achieving equilibrium. Conversely, the palmitoylated calnexin form, where a palmitoyl group was added to cysteine 195, exhibited faster equilibration at around 100 ns and maintained its stability for an extended period of 500 ns within a confined region of roughly 1.5 nm. This observation suggests that the presence of the palmitoyl group enhances the structural stability of calnexin, potentially influencing its function within the endoplasmic reticulum (ER).

To assess regional stability in calnexin, we used root mean square fluctuation (RMSF) analysis, which measures how much individual atoms deviate from their average position over time. Higher RMSF values indicate more flexibility, while lower values indicate more rigidity, which helps to map out regions of structural stability and adaptability.[23] Figure 3 highlights distinct variations in flexibility, particularly in region R1, which corresponds to the area affected by the palmitoyl tail. This increase in flexibility near R1 is notable, as it may modulate the behaviour of calnexin's luminal domain in response to its palmitoylation status. The glycan-binding domain of calnexin, which relies on specific residues for substrate recognition, is of particular interest here. Asparagine at position 293 (N293) in region R2 and lysine at position 330 (K330) in region R3 play key roles in glycan binding and structural stability.

Our findings suggest that the palmitoylation at cysteine 195 increases flexibility around these residues, potentially altering the structure of N293 and K330. Such changes could disrupt glycan recognition and binding, a critical function in ensuring proper glycoprotein folding and quality control in the ER. RMSF analysis further showed that flexibility is primarily contributed by R3, with R1 and R2 providing secondary but significant contributions. This structural flexibility is crucial for calnexin's role as a chaperone, as it allows the protein to adjust dynamically, interacting effectively with a range of glycoproteins and misfolded proteins.

By enabling such dynamic adjustments, palmitoylation enhances calnexin's capacity to maintain ER protein quality control, highlighting the functional significance of lipid modifications in modulating chaperone activity through

altered structural dynamics. This enhanced flexibility supports calnexin's adaptability within the ER, underscoring the role of post-translational modifications like palmitoylation in protein stability and function.

Palmitoylation inducing a conformational change in Calnexin: The functionality of proteins is intricately linked to their structural properties, particularly compactness and flexibility. These attributes can be quantitatively assessed using metrics such as the Radius of Gyration (Rg) and Solvent Accessible Surface Area (SASA). In our study, we explored these parameters for both palmitoylated and non-palmitoylated forms of calnexin, a crucial chaperone protein involved in the endoplasmic reticulum (ER). Figure 4 illustrates the results obtained from GROMACS simulations, depicting the SASA and Rg of calnexin. For the non-palmitoylated form, the Rg peaks at 2.815 nm were accompanied by SASA of 235.4 Å^2. These metrics establish baseline values for assessing the structural impact of palmitoylation.

Following the modification, calnexin's Rg increases to 3.002 nm and the SASA rises to 238.3 Å^2, indicating a slight conformational expansion. This increase suggests that palmitoylated calnexin is marginally less compact and exhibits a greater surface area accessible to solvents. The modest elevation in both Rg and SASA signifies that palmitoylation minimally alters the overall structure of calnexin. However, the functional implications of this slight expansion cannot be overlooked. The increase in surface area may enhance calnexin's dynamic interactions with other ER-associated proteins and substrates, which are essential for glycoprotein folding and quality control processes. Such interactions are critical for maintaining cellular homeostasis, particularly under stress conditions.

Moreover, the observed minor conformational adjustments suggest enhanced flexibility and adaptability in palmitoylated calnexin, that are advantageous for its role as a chaperone. The ability to accommodate various substrates and partner proteins is crucial for effective chaperone activity within the ER environment. Supporting this, the root mean square fluctuation (RMSF) data further confirm that while palmitoylation does not drastically affect the compactness of the protein, it does promote structural adaptability.

To further elucidate the mechanisms behind the observed increase in flexibility, compactness and bulkiness of palmitoylated calnexin, we conducted a thorough examination of the stability and dynamics of its secondary structural units. As depicted in figure 5(a), we analysed the initial conformation of palmitoylated calnexin alongside its time-averaged structures generated from molecular dynamics (MD) simulations. This comparative analysis provides insights into the structural adaptations that accompany palmitoylation.

Utilizing the DSSP module within GROMACS, we closely monitored the temporal evolution of the secondary structural elements in palmitoylated calnexin. The results, illustrated in figure 5(b), reveal distinct alterations in secondary structure, particularly in specific regions of the protein. These structural changes are vital for understanding how palmitoylation impacts calnexin's overall stability and functionality.

One significant observation from the MD simulations is the palmitoyl-dependent orientation of the cytosolic tail relative to the helical axis of the protein.[19] This unique orientation suggests a potential regulatory role for the palmitoyl group, which warrants further investigation to understand its implications on calnexin's function. The R1 region in the root mean square fluctuation (RMSF) graph distinctly illustrates this phenomenon, highlighting fluctuations attributed to the covalent attachment of the palmitoyl tail to a cysteine residue. The interaction between the palmitoyl group and the protein's structure appears to enhance the overall conformational dynamics.

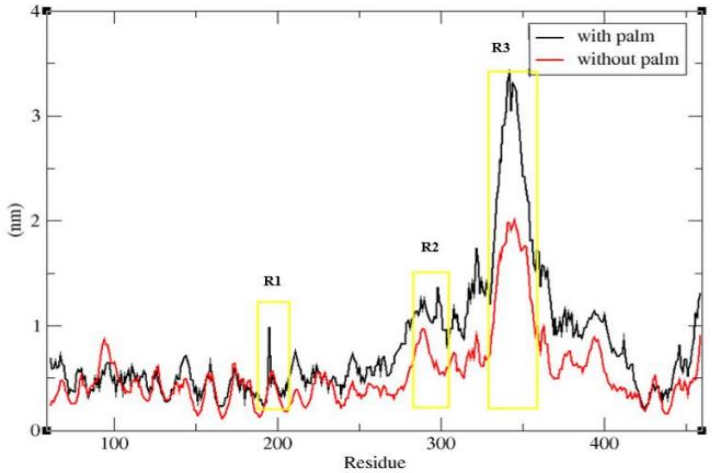

Figure 3: The Root Mean Square Fluctuation graph of topological domain of Calnexin with and without palmitoylation

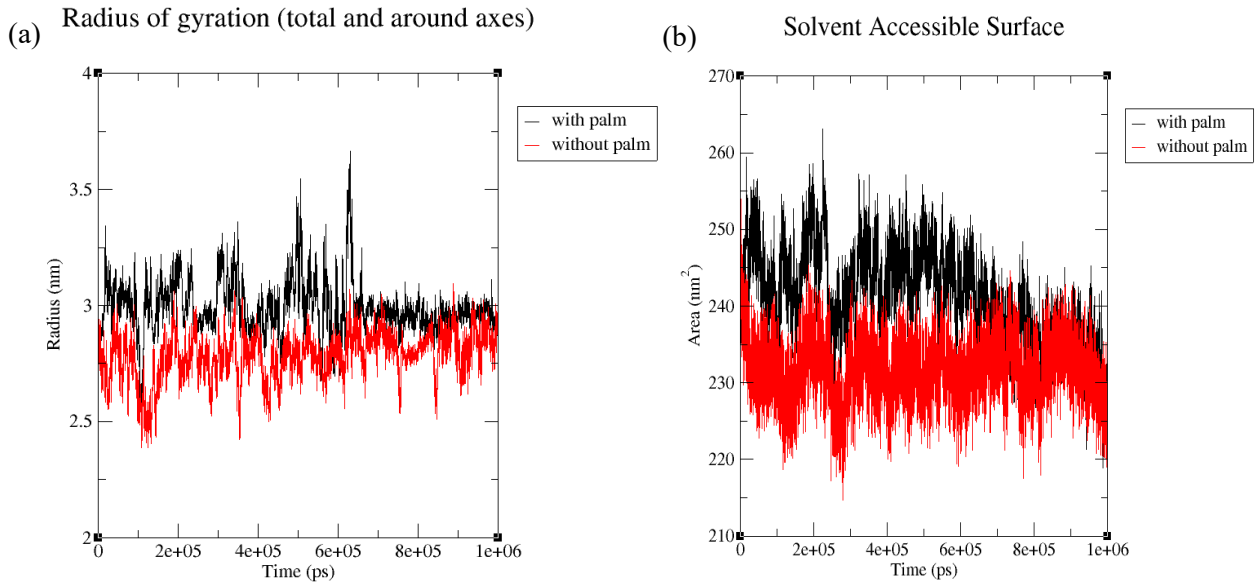

Figure 4: (a) The Radius of Gyration (RG) of topological domain of Calnexin with and without palmitoylation. (b) Solvent Accessible Surface Area (SASA) analysis reveals hydrophobicity differences in the topological domain of Calnexin with and without palmitoylation

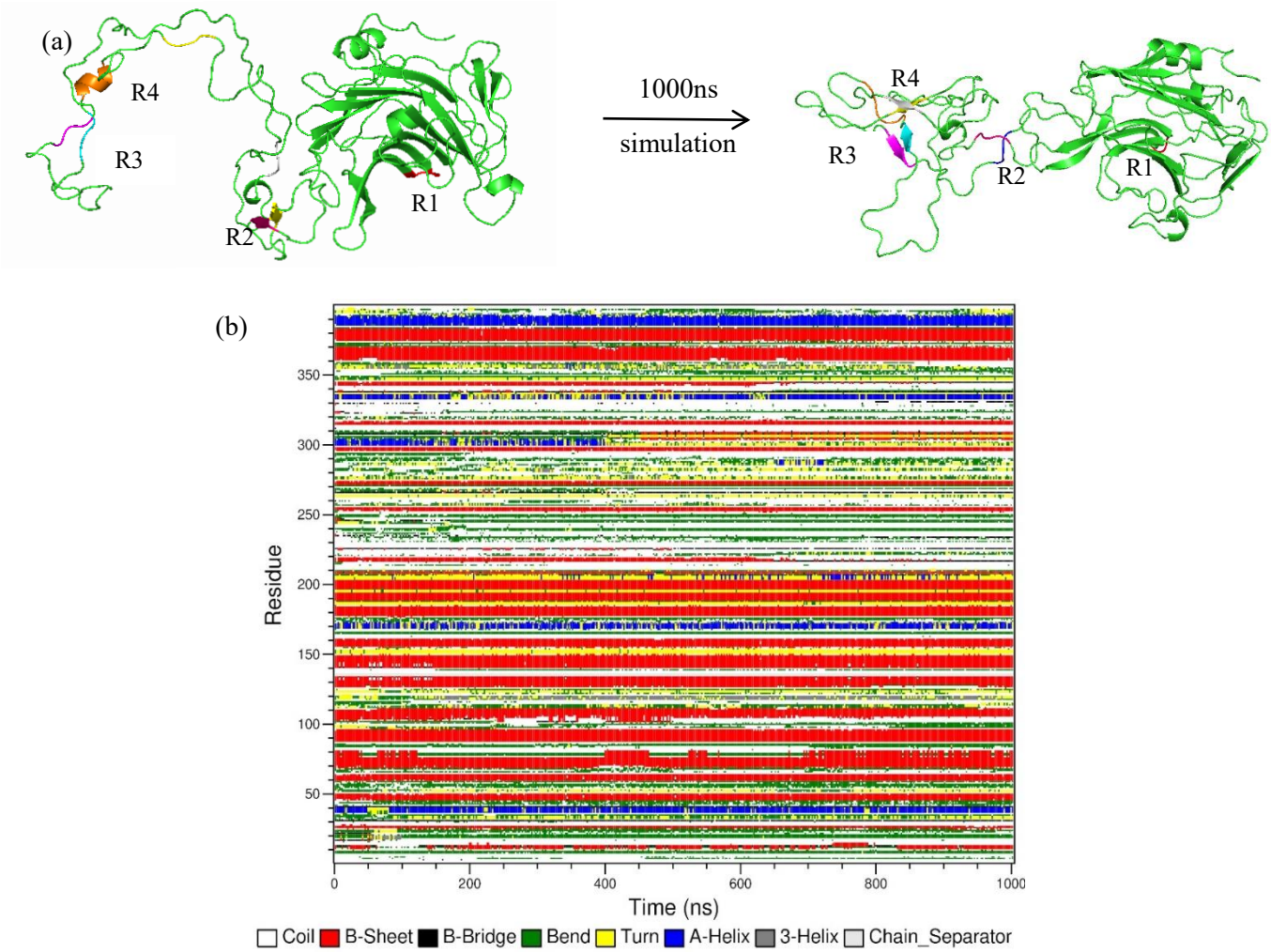

Figure 5: (a)Time averaged structure of Calnexin with palmitoylation compared with initial structure. (b) The time evolution of secondary structural units in Calnexin are shown. The DSSP method was employed to assess the secondary structure content, utilizing the following classifications: bend (green), turn (yellow), α-helix (blue), β-sheet (red), β-bridge (black), coil (white) and 3-10 helix (grey). The distorted regions are marked as R1 through R4.

Moreover, our findings indicate that palmitoylation significantly affects calnexin's conformational landscape, thereby influencing its ability to interact with cytosolic proteins. In particular, the R2 region of the RMSF graph indicates a transition of a beta-sheet structure into a loop conformation, underscoring the structural rearrangements that occur upon palmitoylation. Such transitions are critical as they can modulate the protein's interactions and functionality. The R3 region of the RMSF graph demonstrates substantial fluctuations, revealing a notable shift from a loop to a sheet conformation around residue 331 as well as a transition from a helix to a sheet at approximately residue 360. These transitions are accompanied by temporal changes in secondary structure, providing further evidence of the dynamic nature of palmitoylated calnexin.

These fluctuations imply that palmitoylation can enhance structural malleability, which is essential for calnexin's role as a chaperone. In addition to these larger-scale conformational changes, minor conformational adjustments were noted in certain areas such as the R4 region. These subtle changes may stem from the structural requirements imposed by the addition of the palmitoyl group, highlighting the localized impact of post-translational modifications. The introduction of a palmitoyl group can induce conformational shifts that affect the secondary or tertiary structure of calnexin, ultimately altering the protein's overall flexibility and shape.

Furthermore, the dynamics and function of calnexin may be influenced by these localized changes in conformation. Such modifications can potentially affect the accessibility or orientation of important functional regions within the protein, thereby altering its interactions with cytosolic partners. This intricate interplay between structural dynamics and protein functionality emphasizes the critical role of palmitoylation in modulating calnexin's activity within the ER, as it enables the protein to maintain its essential chaperone functions while adapting to changing cellular environments.[27]

PCA: To measure and understand the differences in motion between native and palmitoylated calnexin structures, essential dynamics analyses were performed. We employed Principal Component Analysis (PCA) using GROMACS tools, specifically "g_anaeig" and "g_covar," to discern changes in the motion patterns of protein complexes. For a more intuitive visualization of these dynamics, we utilized DYNAMITE[28] software to generate "porcupine plots," as shown in figures 6(a) and 6(b). These plots depict arrows on the protein complexes that illustrate both the direction and intensity of movements, offering a clear representation of the differing motion characteristics between palmitoylated and non-palmitoylated calnexin.

Our analysis revealed distinct differences in motion dynamics when comparing the palmitoylated structure to its non-palmitoylated counterpart. The principal components (PCs) obtained from the PCA highlight directions in conformational space that exhibit maximum variability across the simulations. For instance, as illustrated in figure 6(a), the first principal component (PC1) is associated with helix reorientation, specifically showing regions R2 and R3 drawing closer together while transitioning region R2 into a β strand, effectively elongating the β2 strand. This reorientation underscores the dynamic nature of calnexin and how palmitoylation influences its conformational landscape.

Furthermore, the second principal component (PC2) reveals that the contribution to conformational variability across all loops is greater from the helical regions compared to β strands as depicted in figure 6(b). This increased flexibility is essential for calnexin's function, enabling the protein to adapt to the structural requirements posed by different ER substrates. Such adaptability is crucial for calnexin's role in glycoprotein folding and quality control, ensuring that it can efficiently assist in the processing of a variety of substrates.

When we mapped these principal components onto the crucial areas defined by PC1 and PC2 in the MD trajectories, we found notable differences in the dynamics of palmitoylated versus non-palmitoylated calnexin (Figure 6c).[16] The fluctuations of the amino acid region 290-330 in palmitoylated calnexin were characterized by longer arrows, indicating a higher degree of motion and conformational changes during the MD simulations. This suggests that palmitoylation significantly enhances the dynamism of calnexin, particularly in the luminal domain.

The increased motion observed in the luminal domain of palmitoylated calnexin suggests a heightened ability to rapidly alternate between various conformational states. This capacity for dynamic transitions is vital for facilitating interactions with a diverse range of glycoproteins and ER-associated proteins. As calnexin engages in these interactions, its conformational adaptability enhances its efficiency in the ER quality control process. This responsiveness ensures that calnexin can effectively bind and release substrates, allowing it to determine which proteins have achieved proper folding before they proceed through the secretory pathway.

Conclusion

In conclusion, we examined the effect of palmitoylation on the conformational states of calnexin, with a particular focus on cysteine 195 within the luminal domain, utilising molecular dynamics simulations, RMSF, Rg and SASA studies. The findings unequivocally demonstrate that palmitoylation at this location has a substantial impact on the structural dynamics of the protein, altering its compactness, solvent accessibility and flexibility. The palmitoyl tail at cysteine 195 causes more variations in the glycan-recognition areas that are essential for the chaperone activity of calnexin.

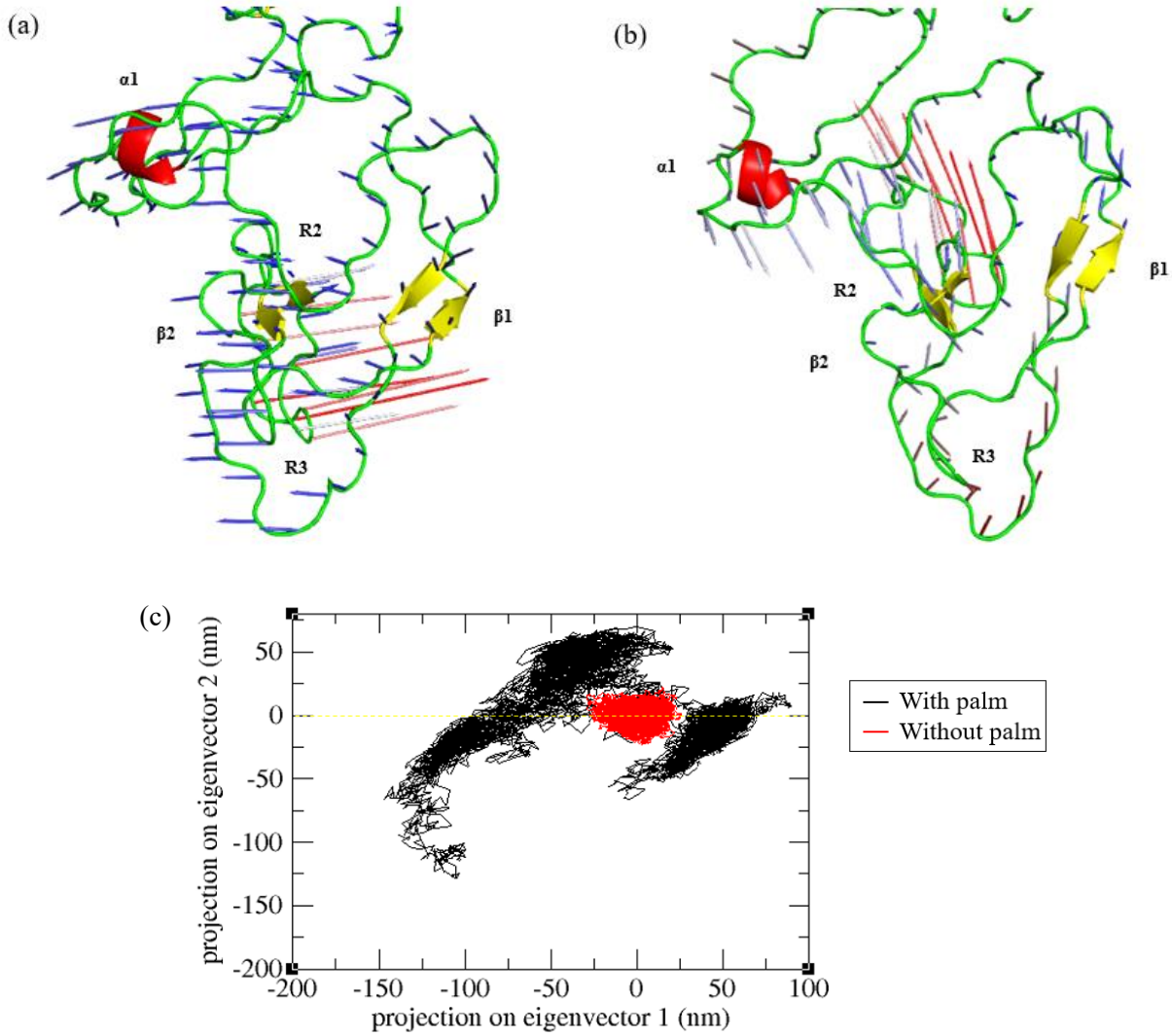

Figure 6: Porcupine plots displaying the conformational changes represented by the first PC and the second PC from the combined ED analysis illustrate the combined essential dynamics analysis (a) and (b). The direction of conformational shift indicated by each PC is indicated by the cone on each Ca atom. (c) From the combined ED analysis, projections of each trajectory along PC1 and PC

As the luminal domain becomes more exposed and structurally flexible, conformational alterations are further highlighted by variations in Rg and SASA values. The functional significance of these small-scale alterations highlights the involvement of palmitoylation at cysteine 195 in regulating the structural flexibility and interaction potential of calnexin.

The efficacy of calnexin in ER protein quality control is supported by palmitoylation at cysteine 195, which enhances its ability to interact with a variety of glycoproteins by encouraging dynamic alterations within the luminal domain. Future investigations into the structural and functional impacts of palmitoylation on calnexin will be made possible by this discovery, which also opens the door for possible connections to protein-folding problems and its implications in ER-associated activities.

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