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Exploring the role of MC1R protein in Zebrafish *Danio rerio* by Molecular Modeling, Dynamics and Simulation Studies

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Abstract: The melanocortin-1 receptor protein (MC1R) is a G-protein-coupled receptor that plays an important role in anti-inflammatory and melanogenic activities, making it a promising therapeutic agent. Considering its functional diversity, we initially focus on predicting Zebrafish MC1R protein structure using EasyModeller v.4.0 to gain structural insights. Next, we describe the structure to highlight the importance of serine-threonine (ST) and aspartic acid-arginine-tyrosine (DRY) motifs, which are crucial for receptor activation and protein stability. Then, we demonstrate the molecular dynamics (MD) simulation of protein in an aqueous environment at 300 and 350 temperatures and present the trajectory analysis of receptor proteins in the dynamic system. We identify that the receptor protein had a significant conformational change with deviations at 300 K at 35 ps and 350 K at 55 ps, respectively. Notably, stability of the receptor protein was obtained at 300 K between 2.7 and 2.8 Å (400 and 600 ps) and at 350 K from 3.1 to 3.2 Å (400 and 600 ps), while this is the first report of the thermostability of the Zebrafish MC1R protein using MD as the main impact. Finally, we discuss how the receptor can be exploited as a therapeutic target for developing new peptides and drugs to improve skin pigmentation and treat pigmentary disorders.

Keywords: MC1R; *Danio rerio*; Zebrafish; molecular dynamics; G-protein.

1. Introduction

Melanocortin receptors are 7-transmembrane G-protein-coupled receptors (GPCR) that belong to the rhodopsin family [1]. Five known members of the melanocortin receptors, MC1R to MC5R, are concerned with different activities in vertebrates [2]. The MC1R gene is located on chromosome number 16q24.3, comprising 4 exons, which gives rise to many intra/ intergenic splice variants [3]. The MC1R is a well-known receptor for α-MSH expressed in the skin and hair follicles, where it controls pigmentation. They are also found in neutrophils, lymphocytes, monocytes, epithelial and endothelial cells [4]. The melanocortin natural agonists are adrenocorticotropin (ACTH) and melanocyte-stimulating hormones (MSH) [5]. The binding of MSH to the MC1R triggers the cyclic AMP (cAMP) response-element binding protein (CREB), which leads to the expression of microphthalmia-associated transcription factor (MITF), thereby resulting in the production of eumelanin [6,7] (Figure 1). In nature, eumelanin has a protective function from the deleterious effects of ultraviolet (UV) radiation

and serves as a scavenger of free radicals. The absence of this pigment makes skin more susceptible to UV damage, a major risk factor in the development of melanoma [8]. MC1R expression is found to be increased in melanomas and melanoma cell lines [9]. Using tissue microarrays, different types of melanomas can be quantified with the help of immunofluorescence and immunohistochemistry techniques to study the expression of the MC1R protein [10]. Consequently, researchers have also identified that there is an association between MC1R and breast cancer. The breast cancer tissue has elevated MC1R expression compared to the normal breast tissue [11].

The low activity of MC1R produces pheomelanin, or the absence of melanin synthesis, and inhibits the MC1R's ability to induce eumelanin production from melanocytes, resulting in fair skin [12,13]. Researchers have demonstrated that the MC1R was primarily concentrated in the cytoplasm and intercellular material of the skin epidermis. Studies revealed an increased level of melanin and tyrosinase activity when α-MSH at 2.5 mg g^{-1} was injected into the caudal vein of red tilapia. The findings confirm that MC1R is involved in melanogenesis when it is bound to α-melanocyte stimulating hormone [14].

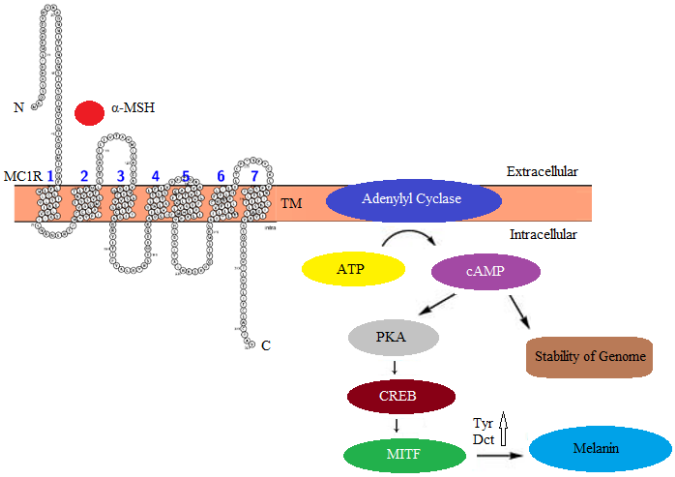

Figure 1. MC1R Signaling pathway in melanogenesis.

These melanin pigments have a principal role in skin coloration through the absorption of UV radiation, thereby facilitating their use as a natural sunscreen. Mutations in the MC1R gene are known to cause changes in the coat coloration of amniotes. In mammals, MC1R is the main receptor protein for agouti-signaling protein, while in fish, it is involved in pigmentation. However, recent investigations reveal that MC1R is widely expressed in different types of cells, such as melanocytes and leukocytes, and is strongly implicated in regulating skin pigmentation, intestinal and ocular inflammation, and inflammation [15,16].

When the MC1R is activated, it exerts a variety of anti-inflammatory and antifibrotic properties. According to a recent study, Dersimelagon (MT-7117) is a bioavailable selective MC1R agonist that can successfully treat erythropoietic protoporphyria [17]. In addition, it is also used to treat scleroderma. The pre-clinical investigations demonstrated disease-modifying effects [18].

Consequently, several researchers have demonstrated the significance of MC1R in immunomodulation through *in vitro* and *in vivo* studies [19].

Additionally, synthetic analogs of *α*-MSH can be used as molecular probes. The most common key motif used in the Structure-Based Virtual Screening (SBVS) technique is His-Phe-Arg-Trp (HFRW) for binding the MC1R protein [20]. Different MC1R agonists are being

developed to enhance pigmentation, oxidative stress, and DNA repair and in the treatment of vitiligo [21,22].

Many experimental studies in aquaculture have found that diets promote fish skin pigmentation. Despite having sufficient data on various feeding strategies to enhance body coloration in fish, research on the molecular aspects of the melanocortin genes remains an enigma. The molecular characterization of the MC1R gene has been described only in some fish, like Oujiang common carp *Cyprinus carpio* var. color, Guppy *Poecilia reticulata*, and the Southern Platyfish *Xiphophorus maculatus* [23-25]. However, there is still much to understand about its role and the regulatory mechanismsmolecular biology, genetics, and developmental biology. The study aims to homology model the MC1R protein and assess that affect fish's differentiation and color variation. In the absence of a crystal structure, it is extremely difficult to access molecular information about the receptor protein, like its interactions and stability. Therefore, there is a need to identify specific receptor proteins that are involved in the melanogenesis process to enhance skin coloration. Thus, it is important to understand the regulation of MC1R for developing lead compounds for therapeutic purposes [26].

In the present study, the Zebrafish *Danio rerio* was selected because it is an emerging model organism in molecular biology, genetics, and developmental biology. The study is aimed at homology modeling of the MC1R protein and assessing the stability of the protein structure under physiological systems by applying molecular dynamics and simulation methods.

2. Materials and Methods

2.1. Sequence retrieval and 3D model building.

The sequence for the MC1R protein was extracted from UniProtKB with ID Q7ZTA3_DANRE (https://www.uniprot.org/uniprot/Q7ZTA3). The three-dimensional structure of the *D. rerio* MC1R protein was searched at the RCSB Protein Data Bank (www.rcsb.org). Due to the non-availability of the crystal structure, a suitable template was searched using the query sequence with the BLASTp suite against Protein Data Bank proteins (PDB) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). The model was built by homology modeling with the help of EasyModeller v4.0 [27].

2.2. Primary structure analysis.

The physical and chemical parameters of the entire *D. rerio* Melanocortin-1 receptor protein were computed by the ProtParam tool (http,//web.expasy.org/protparam/), which was analyzed for molecular weight, theoretical iso-electric point (pI), and grand average of hydropathicity (GRAVY) (www.expasy.ch).

2.3. Secondary structure analysis.

The SOPMA server was used for the secondary structure prediction of the MC1R protein [28]. It was used to assess the conformational information about the positional possibilities of the α-helices, β-strands, turns, random coils, and coils within the protein structure. The transmembrane topology of the receptor protein was predicted with the help of the PSIPRED server [29].

2.4. Homology modeling of the D. rerio MC1R protein.

To find a suitable template for comparative or homology modeling, a BLASTp search of the MC1R protein using the default parameter was conducted against the Brook Haven Protein Data Bank (PDB) (http://blast.ncbi.nlm.nih.gov/). The PDB structures based on identity and sequence coverage were retrieved in PDB format for further investigation. The theoretical structure of the *D. rerio* MC1R protein was predicted using EasyModeller v.4.0. The software generated several preliminary models. The model with the lowest Discrete Optimization of Protein Energy (DOPE) score was chosen for refinement and validation. The modeled structure was visualized using the PyMOL molecular graphics viewer v.2.2.0 (www.pymol.org).

2.5. Model validation of the D. rerio MC1R protein.

The newly generated three-dimensional structure was verified by PROCHECK, which provides information on the stereochemical quality of the protein structure obtained from the Ramachandran plot [30]. The quality of the structure was further investigated using ERRAT. ERRAT is a protein structure verification algorithm ideal for evaluating the progress of crystallographic model building and refinement [31].

2.6. Molecular dynamics and simulation of the D. rerio MC1R protein.

The MC1R protein model was subjected to molecular dynamics simulation in water with TP3BOX size 10 Angstroms by applying ff99sbildn AMBER force fields. For 200 picoseconds (ps), the two equilibration phases of the NPT ensemble with a constant temperature of 300 K, a coupling constant of 1 ps, and the NPT ensemble were applied to all the molecules. After the system equilibration, MD simulations were carried out at 300 K and 350K temperatures for 600 ps using the NPT simulation ensemble method (isothermal-isobaric ensemble). The RMSD (root mean square deviation), RMSF (root mean square fluctuation), and potential energy value of the theoretical model protein were calculated using ProWaVe (https://www.prowave.org/). ProWave is a web server that provides an automated and user-friendly interface for obtaining the solvation-free energy of a protein, which is computed based on the molecular theory of solvation. The solvation-free energy of a protein is the Gibbs free energy change associated with the solvation process, i.e., transferring a protein from a vacuum phase (an isolated protein) to a solution phase (a protein surrounded by water and ions). The MD Run trajectories were analyzed for Carbon Alpha backbone RMSD, RMSF, and total energy using ProWaVe.

3. Results and Discussion

In the present study, molecular modeling of the *D. rerio* MC1R protein has demonstrated the structural and functional components of the receptor protein. The receptor protein is indispensable for body coloration in fish and other vertebrates. Melanism is achieved by the activity of MC1R. When the receptor protein is hyperactive, it leads to the synthesis of eumelanin (brown and black colors), and when it is hypoactive, it produces pheomelanin (red, yellow, and orange colors).

The MC1R protein of *D. rerio* was predicted to have a molecular weight of 36.6 kDa. The theoretical pI was found to be 8.24, which indicates that the protein is positively charged.

The protein's Grand Average of Hydropathicity (GRAVY) value of 0.801 indicates that it is hydrophobic. The secondary structure of the entire receptor protein sequence was found to be rich in the α-helix region with a percentage of 54.49 amino acid residues, and the remaining 16.41 and 26.32 are represented as extended strands and coils. It is quite evident from this secondary structure prediction that the receptor protein belongs to the α-class of proteins. The PSIPRED server predicted the amino acid sequence positions from 7-26, 36-64, 79-107, 120-144, 150-175, 200-225, and 239-258 are probable transmembrane helical regions of the receptor protein (Figure 2).

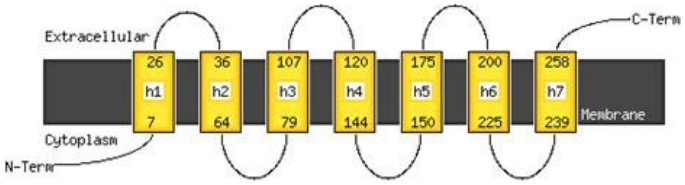

Figure 2. Topology of the *D. rerio* MC1R protein showing 7-TM helices passing through the cell membrane in a serpentine fashion.

Blastp search results revealed 7F4D_R, 7PIU_R, and 7AUE_R as potential templates with 58.42, 57.91, and 57.11% of sequence identity with the query sequence to determine the three-dimensional structure of the receptor protein. The ideal template structure for homology modeling was PDB ID, 7F4D chain R [32]. The three-dimensional structure was generated using EasyModeller v.4.0. The topology of the MC1R protein structure is represented in a cartoon form (Figure 3).

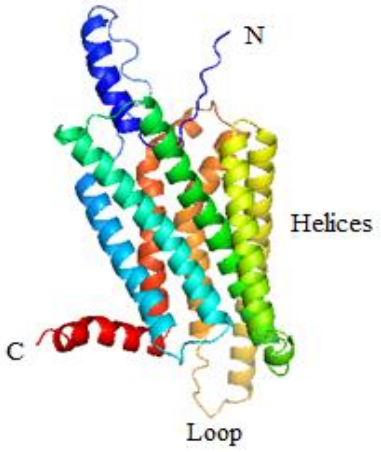

Figure 3. *D. rerio* MC1R protein structure showing N and C terminal regions, α- helices, and intracellular and extracellular loops.

To authenticate the predicted model, the coordinates of both the predicted structure and template (PDB ID, 7F4D_R) were fed into the ERRAT Protein Verification Server. The overall quality factor was found to be 86.59, which is very satisfactory. The validation of the model was further discerned using Ramachandran plot calculations retrieved from the PROCHECK program. The Φ and Ψ distributions of Ramachandran plot analysis revealed that 92.6% and 7.0% amino acid residues are present in the most favored. Additionally, they allowed regions, while 0.4% and 0.0% amino acid residues are in the generously allowed and disallowed regions, which indicates the geometrical fitness of the modeled receptor protein. Altogether, 100% of the residues were in the core and allowed regions. The overall G-factor used was computed as -0.05 (Figure 4).

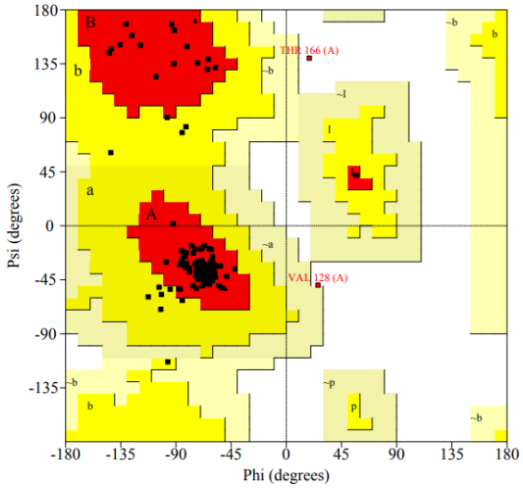

Figure 4. Ramachandran plot for the modeled *D. rerio* MC1R protein.

The modeled receptor protein was identified as a monomer characterized by seven α-helices running along the transmembrane domains from the N-terminal to the C-terminal region, which are aligned parallel to each other. The receptor protein is comprised of 7-TM helices that pass through the cell membrane in a serpentine fashion and are held together by three extracellular and three intracellular loops. It was also observed that the extracellular loops are smaller than the intracellular loops based on their amino acid number.

The α-helices of the monomer located on the extracellular region α1- α6 contain amino acids ranging from 26–36, 107–120, 175-200, and 258–314 and form the outer surface of the receptor protein, called the accessible molecular surface or solvent-exposed area. The three loops of the monomer connect the helices α1- α6 (extracellular) while the other three loops connect α2-α7 (intracellular) respectively. The amino acid serine-threonine (ST), positioned between 30 and 31, is identified as an 'ST motif' of the extracellular loop, which connects α1-α2 helices. While the amino acids aspartic acid, arginine, and tyrosine ranging from 146–148 constitute the 'DRY' motif of the intracellular loop between α4–α5 helices. The helices α2-α7 bearing amino acids from 1–7, 64–79, 144–150, and 225-239 are positioned toward the intracellular or cytoplasmic region of the cell (Figure 2). The present findings are in accordance with the characteristic features exhibited by all melanocortin receptors [33]. The intracellular loops are involved in binding to the G-proteins and facilitate the regulation of signaling, internalization, and cycling. The extracellular loop of the predicted model has a serine-threonine motif critical for protein folding and the stability of the α2 helix TM domain [34]. Both serine and threonine are commonly found polar residues in TM helices involved in backbone hydrogen bonding and facilitating conformational changes in membrane proteins, thereby enabling protein stability [35].

The receptor protein's internal loop has a 'DRY' motif, identified as a conserved amino acid sequence concord with the template (PDB ID, 7F4D_R) involved in ligand-induced receptor activation. In addition, the third loop is unusually conserved and rich in proline and cysteine residues, conforming to a specialized function [36]. Additionally, researchers have shown the presence of a highly conserved DRYxxI/V motif in the intracellular loop of the MC3R sequence. Consequently, it was revealed that the amino acid motif has a significant role in ligand-induced receptor activation. However, under mutations, the MC3R completely lacks or retains partial signaling [37].

Several studies have demonstrated that zebrafish quickly change their pigmentation in response to environmental stimuli. The appearance of black pigmentation is because of the

aggregation or dispersion of melanin within the cell. Aggregation and dispersion are regulated by intracellular cAMP levels [38]. Furthermore, the regulation of MC1R activity is dependent on the intracellular phosphorylation sites. However, mutations in the receptor protein can interfere with the dorsoventral countershading in teleost fish and the skin lightening of the animals. The dorsal side of the fish exhibits an increase in chromatophores, such as xanthophores and melanophores, whereas the ventral region of MC1R mutants shows a decrease in iridophores [39].

Although MC1R exhibits various protective functions, several factors can cause abnormal skin pigmentation [40]. With its distinct skin structure and melanin synthesis mechanism, the zebrafish provides many advantages as a model organism [41]. About 87% of zebrafish genes show high similarity with human genes. Therefore, the melanogenic pathways concerned with the biosynthesis of human melanin are well-conserved [42]. Similarly, genetic variations in the human MC1R gene have resulted in the appearance of oculocutaneous albinism type 2 and increased the chances of developing melanoma [43]. At the same time, the destruction of melanocytes may lead to vitiligo, a pigmentation disorder. The damage caused to the MC1R hampers the synthesis of melanin, which is vital for normal pigmentation [44]. *In silico* analyses demonstrated that Cys35Tyr, Ile155Thr, and Pro256Ser, found in the admixed population of Rio de Janeiro, have a negative effect on receptor function, probably due to changes in the receptor structure. Notably, Cys35Tyr mutation could potentially impair agonist binding, which helps to understand the genetic basis of color variation (Neitzke) [45].

The C-terminal region of the receptor protein has a significant role in coupling, transport, and binding to the plasma membrane [46]. In addition, the GPCR domain of the MC1R protein has been extensively used in the health sector for targeting novel drugs and conducting clinical trials [47].

In the present study, the total energy was found to be -19756 kJ/mol, indicating that the theoretical model is energetically stable when compared to the human MC1R protein with its ligand (EEKE), where the total energy was found to be higher with −1514000 kJ/mol [48]. MD simulations were performed in water to verify the structural changes of the modeled receptor protein. The trajectory analysis was calculated for root mean square deviation (RMSD), root mean square fluctuations (RMSF), and potential total energy. In the RMSD-based results with backbone atoms (Figure 5), the apo state of the receptor protein was observed to be stable from the beginning of the MD run with a deviation of 0-35 ps, and the RMSD value oscillates between 2.0 A and 2.6 Å at 100 ps and 400 ps. However, this follows a relatively stable temperature fluctuation of about 300 K at the production simulation. The protein was stable between 2.7 Å and 2.8 Å at 400 ps and 600 ps.

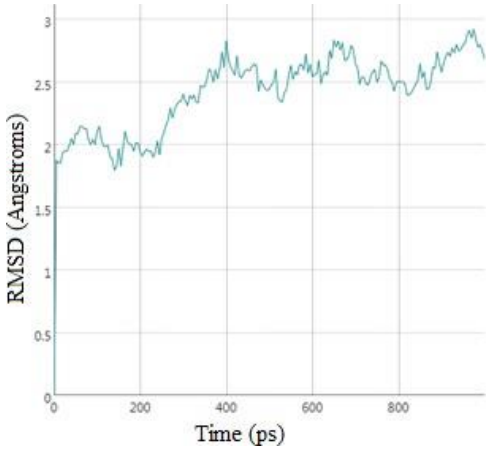

Figure 5. RMSD of backbone atoms for apo state MC1R protein (300 k).

On the contrary, MD simulation with 350K has shown a deviation at 0-55 ps, and the RMSD value oscillates between 2.8 A and 3.0 Å at 100 ps and 400 ps. The protein exhibited stability between 3.1 A and 3.2 A at 400 ps and 600 ps (Figure 6).

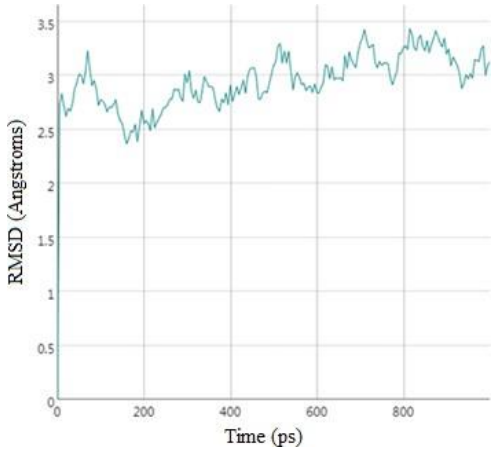

Figure 6. RMSD of backbone atoms for apo state MC1R protein (350 k).

The RMSD fluctuation plot shows the C-alpha backbone deviation during the simulation process at 300 and 350 K temperatures is within the range of 1.5 to 4.5 Å. Since the above results fall within a tolerable range, the stability of the model protein is confirmed. MD simulation at 300 K has displayed the drifts during the entire MD run in the array of 1.9 and 2.7 A, while the plot of MD simulation at 350 K showed drifts from 2.7 to 3.1 Å.

The structural convergence includes terms like RMSD and RMSF. In the apo state, the receptor protein is stable right from the launch of the MD run, with a drift of 0-35 ps at 300 K, followed by a stable confirmation of 2.8 Å until the finale of the entire run. In the case of MD run at 350 K, there was no deviation until 55 ps, while it exhibited an elevated path afterward and extended the RMSD value to virtually 3.2 Å. The deviation in both cases is due to the linear increase in temperature while heating.

The RMSF results display many peaks in the amino acid residues with Cα atoms. At 300 K, residues showed fluctuations from 190 to 226 belonging to the N/C terminal regions of the receptor protein. The protein residues of the receptor were found to be stable, with a near RMSF value of 3 Å (Figure 7).

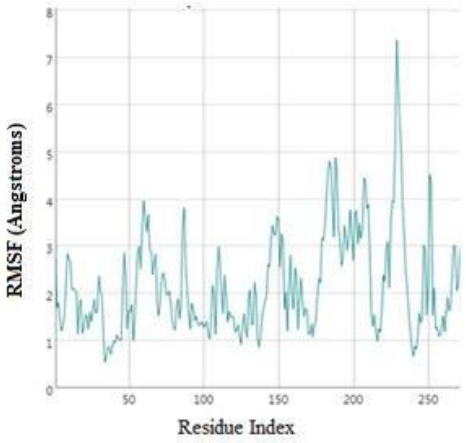

Figure 7. RMSF of Cα atoms for apo state MC1R protein (300 k).

Whereas at 350 K, the peaks were found to increase between 225 and 250 residues due to the influence of temperature. The rest of the entire receptor protein residues were found to be stable, with a near RMSF value of 4 Å (Figure 8).

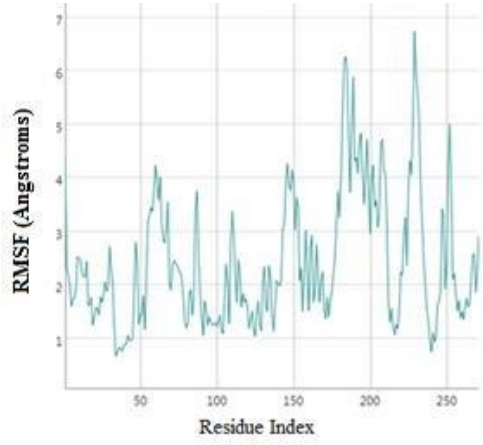

Figure 8. RMSF of Cα atoms for apo state MC1R protein (350 k).

The RMSF results showed many oscillations in the residues with Ca atoms were observed in the case of 300 K, with a fall and amendment in the initial peak due to the influence of temperature on the entire receptor protein, with several numbers of residual fluctuations with a near RMSF value of 3 Å. The same plot also disclosed the presence of a second-high peak in the amino acid residues, leaving most of the other residues stable. However, comparing RMSF outcomes exhibited small variations between 300 and 350 K MD simulations.

Therefore, it is evident that the modeled MC1R protein at 300 K has some structural compactness compared to the 350 K temperature. MD run at 300 K has displayed the drifts during the entire MD run in the array of 1.9 and 2.7 Å. But at 350 K, the plot revealed its stability, with a little drift at 55 ps until the RMSD values oscillated between 2.7 and 3.1 Å. The main purpose of MD simulations is to analyze the structure of proteins at a minimum nano- to micro-second scale to study protein stability, protein function, and protein-ligand interactions [49]. Thereby facilitating the development of peptide-based drug design and enabling selectivity for protein receptors [50]. Moreover, the MC1R agonists/antagonists can be used to control the signaling of receptor proteins, which may represent a therapeutic strategy. Future research on gene editing techniques could be employed to predict gene mutations in abnormal human pigmentation with the help of zebrafish models to study the development of disease and the treatment of pigmentary disorders [52].

4. Conclusions

The use of computational-based approaches has become indispensable to describe the structure and function of a compound at the molecular level. The MC1R protein has been used in research as a potential therapeutic agent for a long time. Several studies have demonstrated the mechanistic pathways of the receptor protein in pigmentation, melanoma, and inflammatory diseases primarily due to physiological variations in melanocytes. Owing to its diverse role, we elucidated the *D.rerio* MC1R protein structure to provide comprehensive structural details. Our analysis revealed that the amino acid motifs, 'DRY' and 'ST', are imperative for ligand-induced receptor activation and maintaining the stability of the α2 helix of the TM domain. Further, the MD simulation study of the apo state of the protein at 300 K and 350 K confirms the stability of the receptor protein. The RMSD and RMSF plots also showed the receptor protein's structural compactness and flexibility. Thus, understanding the structure of the MC1R protein is crucial for the future development of new compounds, peptides, and drugs intended to cure and inhibit certain rare diseases and disorders.

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Conflicts of Interest

The authors declare no conflict of interest.

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