CHAPTER 6

BIOPHYSICS

Doctoral Theses

01. ANCHAL
In Silico Analysis and Functional Characterization of Archaeal FKBP.
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Abstract

The major conclusion which are inferred from this study are as follows: In 2019, all available archaeal FKBPs sequences were collated and analysed by in-silico methods. One short type FKBP was selected as a representative for detailed characterization along with its two mutants. The completely sequenced 196 archaeal genomes were retrieved from NCBI. These retrieved genomes have 325 FKBP sequences consisting of 200 long and 125 short-type FKBP. Longtype FKBPs are present in all five phylum of archaea namely: Euryarchaeota, Crenarchaeota, Thaumarchaeota, Korarchaeota and Nanoarchaeota. Phylum Crenarchaeota, Nanoarchaeota and Korarchaeota and Thaumarchaeota (with exception in an unclassified Thaumarchaeota) have single copy of long-type FKBP only while phylum Euryarchaeota have multiple copies of long and short type homologs. Short-type FKBP are present only in phylum Euryarchaeota. To know the evolutionary diversification of FKBP in archaea, phylogenetic tree was constructed. It was observed that phylogenetic tree of total FKBP sequences divides into two clades, long-type and short-type FKBP. In long-type FKBP tree it was observed that it divided into two clades; the FKBP orthologs from organisms of phylum Euryarchaeota and Nanoarchaeota lie in one clade, while those from phylum Crenarchaeota, Thaumarchaeota and Korarchaeota in the other clade. In short-type FKBP phylogenetic tree, FKBP orthologs from classes of phylum Euryarchaeota was divided in a clade specific manner. Beside this, it was observed that class Halobacteria in phylum Euryarchaeota forms a separate clade in both long and short type FKBP. So, to study short-type FKBP which are unique to archaea, a representative organism was chosen (Haloferax mediterranei from Halobacteria class). The predicted motifs overlap in long and short-type FKBP when compared and suggest that most of the functionally essential residues lie in the N-terminal of long-type FKBP, while Cterminal lacks any conservation of amino acid residues. Two novel motifs were predicted in long-type FKBP: LM1 and LM2, motif LM2 also has a small conserved stretch “DTTXXXXA”. All the active site residues and conserved glycine are predicted within these motifs. In short-type FKBPs, active site residues lie in the predicted motifs and conserved Chapter 8 181 glycine residue lies in SM4 motif. The role of this conserved glycine has not been determined yet and need to be explored. The void of structural information of Haloferax mediterranei FKBP (HmFKBP) has led us to model the structure of HmFKBP. The structure of HmFKBP and its mutant was modelled using MODELLAR 9.14 taking the template Methanocaldococcus jannaschii (PDB ID: 3PR9_A) for homology modelling which share 37% sequence similarity. The modelled structure has similar fold as in archaeal FKBP with four antiparallel β-sheets and two αhelices. On detailed structural analysis of FKBP from various kingdom, it was observed that all groups had variations in active site residues and their conformations. Three amino acid Y26, D37 and R42 has been defined as triad and is reported to form bonds which determine the binding of substrate in human FKBP12. In this triad is Y26 is

conserved in all compared organisms, there is a substitution of D37 (aspartic acid) by asparagine in HmFKBP only, while R42 is least conserved. Tryptophan (W59), which is reported in human FKBP12 as an important residue that changes its orientation during substrate binding, is replaced by leucine in HmFKBP and bacterial orthologs. Biophysical and biochemical characterization of HmFKBP and its mutants (HmF52WFKBP and HmF52W/G87AFKBP) was performed. All of three proteins posses PPIase activity and have chaperone activity but with the variations. HmFKBP have catalytic efficiency of 141 µM -1 s -1 while it is 14.8 µM -1 s -1 and 70 µM -1 s -1 for HmF52WFKBP and HmF52W/G87AFKBP respectively. For chaperone activity, HmFKBP can prevent upto 60 % aggregation of Citrate synthase (CS) while HmF52WFKBP and HmF52W/G87AFKBP could only prevent 37% and 23% of thermal aggregation. All the three proteins possess the ability to prevent the formation of β-amyloid of human β (1-42) peptide. Biophysical characterization of HmFKBP, HmF52WFKBP and HmF52W/G87AFKBP were performed using CD and fluorescence spectroscopy. The λmax observed for HmFKBP, HmF52WFKBP and HmF52W/G87AFKBP is 341, 366 and 358 nm respectively. The thermal unfolding studies of HmFKBP reveals that HmFKBP is a thermostable protein and there are no significant variations in conformation on increasing the temperature from 25 0C to 90 0C. On cooling (90-25 0C), protein refolds back and attains its native conformation Chapter 8 182 with retention of chaperone activity. In HmF52WFKBP, there are structural variations on increasing temperature and upon cooling the protein attains a conformation which is different from the native, hence HmF52WFKBP is not as stable as HmFKBP. In HmF52W/G87AFKBP, it was revealed that on increasing temperature, there is conformational variation but upon cooling the protein acquires the conformation same to the native. This predicts that there is some loss in structure upon heating when is regained upon cooling (90-25 0C). Chemical induced denaturation was performed using urea and GdnHCl and the structural changes were monitored using CD and Fluorescence spectroscopy. In HmFKBP, it was observed that it is stable till 1.5 M urea and after that there is a transition phase till 5 M and after 5 M it is unfolded. In fluorescence spectroscopy with increasing concentration of urea after 1.5 M there is decrease in intensity with constant λmax. On refolding the protein from higher concentration to lower concentration of urea, it was observed that there is gain in negative ellipticity signifying the gain in structure of protein and there is increase in intensity during fluorescence. There are more significant changes in secondary structure than in the tertiary structure with increasing urea concentration but the protein has the ability for reversible folding. In HmF52WFKBP, a characteristic negative ellipticity at 200 nm with an increasing urea concentration was observed along with ellipticity at 222 nm. In CD, on increasing urea concentration after 0.5 M, there is decreasing in its intensity and wavelength shift to around 210 nm from 200 nm. After 4 M concentration of urea intensity of ellipticity and wavelength further decreases and wavelength shifts to around 218 nm from 200 nm. In fluorescence studies, the intensity starts increasing from 0.5 M and increases uniformly till 2.5 M concentration of urea. After 2.5 M it increases slightly till 5 M and after 5 M it further increases with slight red shift in wavelength. This increasing intensity with red shift depicts the unfolding of HmF52WFKBP on increasing the concentration of urea. HmF52WFKBP starts losing it conformation after 0.5 M concentration of urea. On refolding, it was observed that HmF52WFKBP also have ability of reversible folding. In HmF52W/G87AFKBP, on increasing the urea concentration, there is no significant change in ellipticity at 222 nm. And in fluorescence there is slight increase in intensity with increasing concentration. This depicts the stability of protein in higher concentration of urea. Chapter 8 183 On GdnHCl induced denaturation, HmFKBP retain its native structure till 1 M and is completely unfolded after 4.5 M concentration of GdnHCl. There is decrease in fluorescence intensity after 1 M GdnHCl and it decrease till 4.5 M and after this it is almost constant. Hence, HmFKBP is moderately stable in GdnHCl. And on refolding, HmFKBP regains its conformation. HmF52WFKBP and HmF52W/G87AFKBP have similar stability in GdnHCl as HmFKBP. In both the mutants there is increase in intensity with increasing

denaturant concentration, which decrease on refolding. Urea and GdnHCl mediated unfolding of HmFKBP and its mutant was also investigated with ANS binding assay. The λmax of ANS with native HmFKBP is around 480 nm. During urea mediated unfolding it remains constant till 1.5 M after this there is shift in wavelength from 480 to 495 nm on reaching 5 M, after 5 M it is 500 nm till 7 M. During GdnHCl mediated unfolding a red shift from 480 nm to 500 nm is observed from native. In HmF52WFKBP, λmax of ANS with native protein is around 488 nm. In urea denaturation, from 1 to 5 M there is red shift from 488 to 510 nm in λmax, and after 5 M it becomes constant at 513 nm till 7 M.in GdnHCl mediated unfolding, there is a red shift from 488 nm to 515 nm from native. In HmF52W/G87AFKBP, λmax of ANS with native protein around 498 nm. On GdnHCl denaturation there is shift in λmax from 498 nm to 519 nm from native. These observed shift in wavelength determines the unfolding of protein and these results are in accordance with CD and Fluorescence spectroscopy observations. The stability of HmFKBP and its mutants was also investigated at different pH from 1 to 10. The changes observed in secondary and tertiary structures states that there are conformational changes in the lower or acidic pH i.e from 1 to 5 in reference to the changes from pH 6 to 10. These changes suggest the instability of protein in that pH. Hence, all three proteins are stable from pH 6-10. The exposure of hydrophobic patches at different pH was investigated with ANS binding assay. It was observed that in pH from 1 to 5 there is increase in intensity of ANS binding due to exposed hydrophobic patches and the conformational changes. While from pH 6-10 the observed intensity is almost constant without much variation in λmax. To validate the results obtained in-vivo and to predict the difference in behaviour of HmFKBP and its mutants we have performed Molecular dynamic simulation. The modelled Chapter 8 184 structures of HmFKBP and its mutants were simulated at 25 0C (298K) for 300 ns to obtain the equilibrated structure. The obtained equilibrated structure was aligned with modelled structure to remove the biasness in the structure. The equilibrated structures were utilized for further studies. The parameters like RMSD, RMSF, SASA and Rg were used to decipher the difference between them. To investigate the thermal stability HmFKBP and its mutant were simulated at various temperatures from 25 to 90 0C (298-363K) for 300 ns. In HmFKBP it was observed that from 25 0C (298K) upto 55 0C (328K), RMSD fluctuations indicating conformational changes were observed upto 25 ns which later get stabilised. However, temperatures at and above 65 0C (338K) exhibit higher RMSD which determines the changes in conformation above 65 0C (338K). In Rg analysis, it was observed that at 65 0C (338K) and 75 0C (348K) the fluctuations in the radius of gyration were higher while below 55 0C (328K), the structure shows compactness. These infers about structural variations in HmFKBP at and above 65 0C (338K). In HmF52WFKBP, it was observed that the RMSD fluctuations were observed at most of the temperature ranges. Variation in RMSF values was observed at 45 0C (318K), 65 0C (338K) and 90 0C (363K) throughout the whole structure. It was observed that Rg values reduced on increasing the temperature at and above 45 0C (318K) while Rg values fluctuations were observed at 35 0C (308K) and 90 0C (363K). This observation indicates that there is conformational variation in protein throughout temperature range. In HmF52W/G87AFKBP, at temperatures at and above 45 0C (318K), the structure undergoes sudden conformational change and RMSD stabilises within 50 ns. RMSF fluctuations were observed at all the temperatures. In Rg values it was observed that except for 35 0C (308K), 55 0C (328K) and 85 0C (358K), at all the temperatures protein experience decrease in Rg values. This infers about continuous structural variation at all temperature. Comparative studies of HmFKBP, HmF52WFKBP and HmF52W/G87AFKBP reveals that at temperature upto 45 0C (318K) the double mutant (HmF52W/G87AFKBP) behaves like wild type (HmFKBP) while at 55 0C (328K), all the three proteins behave in similar manner and from 65 0C (338K) to 85 0C (358K) HmF52W/G87AFKBP behaves like single mutant HmF52WFKBP. Chapter 8 185 It has been established in our study that short type FKBP from halobacteria and its mutants not only exhibits PPIase activity with variation in its catalytic efficiency but also prevent thermal aggregation of target protein and prevent

formation of β-amyloid thus, possess chaperone activity. However, there are structural variations among them even with single point mutation which effects their thermal and chemical stability.

Contents

1. Introduction 2. Review of literature 3. Masterials and methods 4. Distribution of archaeal FKBPs. Sequence and structure analysis 5. Biochemical and biophysical characterization of short-type archaeal FKBP from haloferax mediterranei (HmFKBP) 6. Biochemical and biophysical characterization of mutants of short-type archaeal FKBP from haloferax mediterrranei (HmFKBP) 7. Thermal studies of haloferax mediterranei (HmFKBP), HmF52WFBP and HmF5W/G87AFKBP using molecular dynamics simulation n8. Conclusions 9. Additional projects undertaken. Refernces. Annexures

02. KOREN (DANIEL TUIKHANG)
Biophysical and Electrophysiological Studies on the Modulation of Voltage-Dependent Anion Channel by Homoeysteine-Thiolactone. Calmodulin, and Calmodulin-Dependent Protein Kinase II.
Supervisor: Prof. Manisha Goel and Dr. Subhendu Ghosh
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Abstract

Mitochondrial membrane damage is often regarded as one of the significant events in mitochondria mediated cell death. Various ligands, metabolites, and proteins are involved in the regulation and maintenance of mitochondria through numerous mechanisms. It is believed that the voltage dependent anion channel. One of the most abundant proteins from the outer mitochondrial membrane, could be their possible target or site of action. The present work aims at the biophysical and electrophysiological studies on the modulation of the gating properties of VDAC from rat brain mitochondria by homocysteine thiolactone, calmodulin, and calmodulin dependent protein kinase. Our bilayer electrophysiology studies suggest that VDAC's function is modulated by HTL.CaM and CaMKII. The voltage dependent nature of VDAC is retained even after it has interacted with HTL or CaM whereas there was a significant loss due to its interaction with CaMKII. However all three molecules HTL CaM and CaMKII signifincatly reduce the conductance of the VDAC single channela t all the holding potentials between 60mV and 60 mV. It exhibits maximum conductance at a lower potential and subsequently reduces to the lowest conducting state of native VDAC at a higher potential. It remained in a single state for a longer duration with no significant closing events. The net gating charge was significantly reduced by HTL,CaM and CaMKIL. Moreover spectrofluorometric and molecular docking studies indicate that HTL and CaM have interacted with the purified VDAC protein. Fluorescence data analysis indicates that the protein ligant protein interaction. VDAC and HTL or CaM is not a simple or linar process ratherit has a nonlinear mode of interaction.

Contents

1. Introduction 2. Experimental procedures and methods 3. Interaction of homoeysteine thiolactone with voltage-dependent anion channel 4. Interaction of calmodulin with voltage dependent anion channel 5. Interaction of calmodulin dependent protein kinase II with voltage dependent anion channel 6. Summary of findings and conclusions. References. Events Participation and Achievements.

03. PANDEY (DEEKSHA)
Characterization and Analysis of Antimierobial Resistance Using In-Silico Tools Haracterization and Analysis of Antimierobial Resistance using in-Silico Tools.
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Abstract

The alarming increase in antimicrobial resistance (AMR) is now a global health crisis, indiscriminate use misuse and overuse of antibiotics have led to the development of AMR in all pathogenic microbes which is regarded as the greatest scourge of the 21st century. AMR can evolve either by gene mutations and or the acquisition of resistance determinants through horizontal gene transfer from other microbes. Antibiotic resistance (AR) is a dynamic phenomenon and has escalated as a significant problem of broad public health significance. In the recent past the world health organization has published a list of bacteria that pose serious threats to human health. These bacteria are categorized as priority pathogens commonly known as ESKAPE pathogens. In bacteria several mechanisms contribute to the development of antibiotic resistance for example evolving mutations in the antibiotic largest modifications in the bacterial cell surface that prevent antibiotics from penetrating inside the cell efflux pumps which pump out the antibiotics from the cell even before they reach their target and producing enzymes which inactivate the antibiotics. Hence regular surveillance of antibiotic resistance genes in microbes and met genomes from human animal and environmental soruces is vital to understanding epidemiology and foreseeing the emergence of new antibiotic resistance determinants. Whole genome sequencing based identification of the microbial using antibiotic resistance databases and in silicon prediction tools can significantly expedite the monitoring and characterization of in various niches. The major hindrance to the annotation of ARGs from WGS data is that most genome databases contain fragmented genes genomes due to incomplete assembly. In this study we have developed freely accessible machine learning based in silico bacterial surveillance tools named bacterial antibiotic resistance scan novel bacterial efflux prediction and β lactamase family prediction for characterization of prediction of bacterial efflux proteins responsible for and identify their corresponding families the prediction and annotation of ambler's class, subclass and families of β lactamases respectively in the comics datasets including short sequencing reads and fragmented contains. The designed tools construct and library of reported to annotate next generation met genomic samples.

Contents

1. Introduction 2. Review of literature 3. Materials and methods 4. An in-sillico resource to discern diversity in antibiotic resistance genes 5. Two-tier system to predict and categorize bacterial efflux mediated antibiotic resistance proteins 6. Prediction and classification of lactamase class, subclass & family 7. Investigating the OXA variants of ESKAPAE pathogens 8. Summary & Future Prospects. Bibliography. Achievements. Annexures.

04. SIDDIQUI (SHUMAILA IQBAL)
Modulation of Passive Diffusion Channels: Biophysical and Electrophysiological Studies on VDAC and Connexin.
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Th 26578

Abstract

The present work aims at the study of the modulation of specific passive diffusion channels, namely voltage dependent anion channel and connexin. These channels are two important types of voltage dependent membrane proteins that play critical roles in the flow of ions across the mitochondria and cell membranes respectively. VDAC also known as mitochondrial porin is a channel residing in the outer membrane of the mitochondria of an eukaryotic cell and has a preference for anions at lower voltages. Other than final control of ATP transport from mitochondria to the cytosol VDSC is known to play an important role in cellular homeostasis oxidative stress apoptosis. On the other hand connexins are non selective channels from the outer membrane of an eukaryotic cell. These proteins are involved in a variety of processes including electrical signaling (especially in excitable cells like neurons cardiac cells) cell-to-cell communication and the related physiological events. Despite the fact that several studies have been conducted to strengthen our understanding of the biophysical and electrophysiological properties of VDAC and cannexins, still a large number of issues regarding this remain unanswered. For example how at a single ion channel level it interacts and overcomes the cellular stress, post translational modification and oxidative agents and the effect of anti oxidants in repairing oxidantion damage. The medthods used for this purpose were bilayer electrophysiology and other biophysical tools. The biology of all the above mentioned processes are complex. Keeping this in view in vitro approaches have been made in the present investigations.

Contents

1. Introduction 2. Materials and methods 3. Phosphorylation of voltage dependent anion channel (VDAC) by extracellular signal regulated kinase 1 (ERK) 4. Voltage dependent anion channel and its interaction with N-acety I L-Cysteine (NAC) under oxidative stress on planar lipid bilayer 6. Interaction of phytochemicals like thymoquinone with connexin 7. Othr membrance proteins acting as passive diffusion lon channels. 8. Summary and Conclusion.