**ABSTRACT**

Mitochondrial Complex-I (NADH: ubiquinone oxidoreductase) is the protein complex that carries out the first step in the respiratory chain process. During cellular respiration, Complex-I transfers two electrons from NADH to ubiquinone with simultaneous pumping of four protons across the inner mitochondrial membrane, generating a proton motive force driving ATP synthase to synthesize ATP. Being a large complex structure, it has many constituent subunits, 44 in eukaryotes and 14 in prokaryotes. This multi-subunit complex is divided into 3 domains: dehydrogenase domain (N module), hydrogenase/ubiquinone reduction domain (Q module) and proton pumping domain (P module). Determining the atomic resolution structure of the entire Complex-I is a daunting task owing to its large size. Due to the absence of information on subunit organization; Complex-I was considered an L-shaped black box. Only recently, the three-dimensional structure of a prokaryotic Complex-I from *Thermus thermophilus* has become available (PDB ID: 2FUG, 4HE8, 4HEA). This structure is a 550 kDa complex consisting of 14 subunits conserved across organisms and some additional accessory subunits. Owing to poor resolution, the individual subunits are not distinguishable in the available eukaryotic Complex-I structure from yeast. Thus, further exploration is required to understand eukaryotic complex, in particular, the human mitochondrial Complex-I.

The subunits of human Complex-I are known mutational hot spots for several disorders. The mutations may be mitochondrial or nuclear in origin resulting in genetic diseases which may be inherited maternally or in an autosomal recessive fashion. The mutations result in a dysfunctional Complex-I causing Complex-I deficit disorders. The symptoms observed range from encephalopathy, cardiomyopathy, neurological disorders or a combination of two or more of these symptoms. Leigh syndrome is one such genetic neurological disorder caused by mutations of Complex-I. Two among every 1,00,000 newborns is affected by this disorder. There is also speculation about the damage that can be caused by reactive oxygen species (ROS) produced when Complex-I is not properly assembled. Only correctly assembled hydrophilic domain can prevent ROS production. Thus, these disorders are thought to be due to disruption of Complex-I assembly by mutations in subunits affecting function and causing damage due to ROS generation. Analyzing the differences
between wild-type (w-t) and mutant subunits would be a step towards understanding at molecular level the potential defects in complex assembly caused by pathogenic mutations and its resultant dysfunction. It would also help associating the broad nature of symptoms specifically to functional deficit of Complex-I in the diseases like Leigh syndrome.

In the present thesis, *in silico* and biophysical approaches have been adopted to study some of the core subunits of the ubiquinone reduction module (Q module) of the human mitochondrial Complex-I. The Q module possesses the iron sulphur cluster N2 vital for its activity. The module acts as a connecting link between the N and P module and assembles during the early stages of Complex-I assembly to form the peripheral arm. The core subunits of Q module are *NDUFS2, 3, 7 & 8*. These subunits are highly conserved in bacteria to mammals; especially their C-terminal part shows an identity of 42% in certain cases. Utilizing the similarity between the human and *Thermus thermophilus* Complex-I subunits, the four core human subunits in the Q module were modelled using molecular modelling based on the *T thermophilus* structure (3I9V, 3IAM, 3FUG) in MODELLER9.10 and PRIME v3.1 of the Schrodinger suite. The mutants causing Leigh syndrome were prepared *in silico* using the FoldX program. The mutations were then classified into those in the interior, those at the interface and those near the binding site depending on their positions in the Q module.

Molecular dynamic simulations were performed in GROMACS v4.5 to understand the effects of these double/point mutations on the structure. The w-t and mutant structures were compared in terms of root mean square fluctuation (RMSF) of residues, radius of gyration (Rg), hydrogen bonding, solvent accessibility etc along a 15 ns trajectory. The substrate n-decyl ubiquinone (n-DBQ) was docked at the interface of *NDUFS2 & 7* using Glide v5.8. Energy calculations were performed in Bioluminate 1.0 to study the effects of subunit mutations on the stability of the Q module, iron sulphur cluster, mutual affinities of subunits and affinity to n-DBQ.

In the dynamic simulation studies *NDUFS2* showed a high RMSF mainly at the location of mutation, whereas *NDUFS3, 7 & 8* showed fluctuations spread over the whole protein. In the case of *NDUFS3 & 8* the w-t proteins were more compact (measured by Rg) compared to mutant proteins. All the mutant subunits were found energetically unstable compared to their w-t counterparts. Some of them showed
reduced affinity to n-DBQ or iron sulphur cluster N2. Although the mutations caused minimum perturbation to the overall secondary structure, the root mean square fluctuations of certain segments were substantial, especially those in the loop regions. The mutations affected the hydrogen bonded interactions, solvent accessible area and the observed radius of gyration to various extents. Molecular dynamic simulations of the \textit{NDUFS2} and 7 complex along with n-DBQ revealed the reduced affinity of the mutants towards n-DBQ. Also, high fluctuations of residues near the n-DBQ binding site was observed in the mutants during MD simulations; indicating that even when the mutation positions are not in the vicinity of binding sites, they certainly influence the binding.

The cumulative effect of all the changes due to mutations on the formation of complex assembly is reflected in the observed unfavourable energy variations, instability of subunit association and a reduced affinity for substrates such as ubiquinone. Thus the analysis indicates that Leigh syndrome mutations lead to formation of structurally and functionally defective complex, which in turn results in disease phenotype. Further attempt was made to observe the nature of the subunits \textit{in vitro} to extrapolate the \textit{in silico} observations in solution.

Although we tried to clone and express all the subunits of Q module, finally we were successful in completing studies only on two subunits, the structural subunit \textit{NDUFS3} and the functional subunit \textit{NDUFS7}, which were cloned and expressed via autoinduction in \textit{E coli}. The full length genes of both the subunits were amplified from the cDNA obtained from RNA of the \textit{HT 29} cell line. The genes were further cloned into pGEM-T vector followed by subcloning in pET28b(+) expression vector between Nde I and Xho I sites and transformed in \textit{BL21DE3} expression cells. The subunits engineered by incorporating mutations identified with Leigh syndrome were prepared by site-directed mutagenesis. A double mutant of \textit{NDUFS3} (T145I+R199W) was prepared by a two step PCR protocol. Two point mutants of \textit{NDUFS7}: V122M and R145H were prepared in a single PCR step. The products of site-directed mutagenesis were ligated and further subcloned in expression vector pET28(+) between Nde I and Xho I sites. The w-t proteins and corresponding mutants were further expressed by autoinduction and purified. \textit{NDUFS3} and its mutant were purified by Q-Sepharose ion-exchange chromatography followed by Sephadex G-200 size exclusion chromatography. \textit{NDUFS7} and its mutants were purified by Ni-NTA
affinity chromatography followed by Superose 12 size exclusion chromatography. The expression and purity of proteins were confirmed by 12% SDS-PAGE and Western blot; molecular weights were determined using MALDI TOF/TOF.

The w-t and mutant proteins have been compared for their stability, aggregation propensities, binding with n-decylubiquinone and other biophysical properties. The mutant proteins of both the subunits showed differences with respect to the corresponding w-t counterparts in secondary and tertiary structure and higher aggregation propensity. Apart from these, mutant *NDUFS3* showed a different native structure as compared to the w-t although the conformational transitions at different pH, temperature and Gdn-HCl concentrations were similar. The tryptophan microenvironment and secondary and tertiary structure of w-t *NDUFS3* were substantially different from that of the double mutant. Despite the point mutants of *NDUFS7* showing a structure similar to the native w-t *NDUFS7* the peak at 325 nm characterising cluster N2 was absent in them. Both the mutants of *NDUFS7* showed reduced binding affinity towards n-DBQ. In the w-t, an increase in the binding affinity to n-DBQ was observed with an increase in temperature, however, the mutants showed an opposite trend of decrease in binding affinity with temperature. Rayleigh scattering, Thioflavin-T and Congo red dye binding studies helped to compare and quantify the differences in aggregation tendency of the proteins. The mutants showed a high aggregation propensity in both *NDUFS3* and 7 as compared to the w-t. At physiological temperature (37°C), an increase in aggregation was observed only in the mutant proteins. A higher aggregation propensity of the mutants would lead to lesser number of molecules available for assembly of Complex-I.

These different properties highlighted the change in the functional nature of the mutant proteins *in vitro*. These changes have been correlated with complex functional defects or complex disassembly observed in Leigh syndrome. Using computational and biophysical approaches, the structural and functional defects in the mutant subunits have been demonstrated. This has contributed to understanding molecular level effects of pathological mutations causing Leigh syndrome.