Review of literature
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All living cells contain many thousands of different proteins, each of which carries out a specific chemical or physical process. Due to the importance of proteins in basic cellular functions, there has been a great interest in studying the process of protein synthesis. Because of intensive research activity on protein synthesis, little attention was paid to the fact that many proteins are rapidly degraded to amino acids. The concept of protein turnover is hardly 60 years old. Earlier, body proteins were viewed as essentially stable constituents that were subject to only minor 'wear and tear'; dietary proteins were believed to function primarily as energy providing fuel, traversing metabolic pathways, which were completely distinct from those of the structural and functional proteins of the body (Ciechanover, 2005). Analyzing the fate of dietary protein was hard to approach experimentally, as research tools were not available, then. Main reason being the lack of main reagent, stable isotopes to track a protein's fate (Ciechanover and Schwartz, 2004). Schoenheimer and co-workers discovered that following the administration of $^{15}$N-labelled tyrosine to rat, only $\sim 50\%$ was recovered in the urine, while most of the remaining was deposited in tissue proteins (Schoenheimer et al., 1939). An equivalent of unlabelled nitrogen was excreted. It was concluded that newly incorporated amino acids must have replaced those in tissue proteins in a process of dynamic protein turnover. They further discovered that from the half that was incorporated into body proteins only a fraction was attached to the original carbon chain, namely tyrosine, while the bulk was distributed over other nitrogenous groups of proteins (Schoenheimer et al., 1939), mostly as $\alpha$-NH$_2$ group in other amino acids. These experiments demonstrated unequivocally that the structural proteins of the body are in a dynamic state of synthesis and degradation and that even individual amino acids are in a state of synthesis and degradation.

Even after these discoveries, there were hardly few people who believed the 'dynamic state' of body structural proteins (Schoenheimer, 1942). However, gradually accumulated experimental evidences indicated that the degradation of intracellular proteins is an important cellular function, which is extensive and selective. It was observed that abnormal proteins produced by the incorporation of some amino acid analogues, were selectively recognized and rapidly degraded in cells. By late 1960s,
experiments proved that normal proteins were also degraded in a highly selective fashion. The half-life of many proteins in the cell was studied and found to be ranging from minutes to days. This may have an important role in maintenance and growth of cell. However, the biochemical system that carried out this process at such a high degree of selectivity and sophistication was not known. With the discovery of lysosome by Christian de Duve (Bowers, 1998), it was assumed that cellular proteins were degraded within this organelle. At that time, it was believed that lysosome was the only organelle responsible for all protein degradation. Yet, the mechanism responsible for such selectivity was not understood.

Avram Hershko, got probably the first clue that protein degradation is an energy dependent process. He observed that the degradation of Tyrosine Amino Transferase (TAT) was completely arrested by potassium fluoride, an inhibitor of cellular energy production (Hershko and Tomkins, 1971), which suggested involvement of some non-lysosomal mediated pathway for protein degradation. Hershko, Ciechanover and Sore, in their Nobel prize winning work, tried to establish a cell free system to study protein degradation. During this study, they identified a low molecular weight protein ATP dependent Proteolysis Factor 1 (APF1), which usually associated with other high molecular weight proteins through covalent amide bond (Hershko, 1996) and the similarity of APF1 with ubiquitin was established later (Wilkinson et al., 1980).

Even though ATP-dependent protein degradation pathway was discovered in the 1970s, involvement of the proteasome particle was demonstrated only in 1989 (Matthews et al., 1989). Proteasomes, the main degradation machinery of cellular proteins, was discovered by a different set of researchers. Wilk and Orlowski (1980) at the Mount Sinai School of medicine found a complex from bovine pituitaries that displayed multicatalytic protease activities. Arrigo et al. (1988) showed that this protease was identical to a particle known as prosome, which was originally discovered by Kalus-Scherres. It was in this paper Arrigo proposed the name Proteasome (Arrigo et al., 1988). Link between the ubiquitination of cellular proteins and degradation by proteasome was established a few years later (Ciechanover et al., 2000).
Protein breakdown not only regulates the amount of intracellular proteins, but is also involved in their quality control. However, proteolysis has been considered for years as a non-selective process responsible for basal protein turnover, the elimination of abnormal proteins and the regulation of some key enzymes by unclear mechanisms. It is now clear that the degradation of cellular proteins is a highly complex, temporarily controlled and finely regulated process that plays major roles in a variety of basic cellular pathways during both cell growth and death (Goldberg, 2003).

From the work of many scientists around the world, we now know that intracellular protein degradation is an important regulatory step in cell growth; is itself a selective process; and there are two major pathways that carry out protein degradation:

i. **Lysosomal**: Proteolysis of endocytosed proteins such as membrane receptors or extra-cellular proteins is largely confined to the lysosomal / vacuolar system.

ii. **Non-Lysosomal**: Energy-dependent proteolysis by proteasomes is the major site for the degradation of cellular proteins and plays important roles in many aspects of cellular regulation.

### 2.1 Degradation of cellular proteins: a regulated process

The regulated degradation of cellular proteins has two main objectives. (i) to remove / degrade unwanted, used up and misfolded proteins from the cell environment, which will otherwise become toxic; and (ii) to regulate important cellular processes by controlling the amount of different functional proteins.

In eukaryotes, the regulated intracellular protein degradation is mainly achieved by Ubiquitin-Proteasome System (UPS) (Rose, 2005). The degradation of a protein via the UPS involves two discrete and successive steps. Protein substrate is first tagged by the covalent attachment of multiple ubiquitin molecules and then degraded by the degradation machine, the 26S proteasome complex, leading to the subsequent release of free, reusable ubiquitin and small degraded peptides. In prokaryotes, a homologous system called HslUV is involved in intracellular protein degradation, but the details of the process are less understood (Rohrwild et al., 1996).
2.1.1 Ubiquitin-proteasome system: a robust, modular system

Ubiquitin proteasome system degrades diverse cellular proteins with exquisite specificity. To accomplish this, highly modular and elaborate machinery is employed. Degradation through UPS pathway has two phases. In the first phase, proteins to be degraded are selected and marked by attaching degradation signal, by a set of processes called as ubiquitin conjugation cascade. Ubiquitin is composed of 76 amino acid residues and its primary sequence is highly conserved from protozoa, to yeast, to archaea to mammals (Schlesinger and Goldstein, 1975). Degradation signal usually consists of multiple chains of ubiquitin. In the second phase, tagged proteins are recognized and degraded by 26S proteasome machinery (Glickman and Ciechanover, 2002).

2.1.1.1 Ubiquitin conjugation cascade: imparts specificity

In the first step of ubiquitin conjugation cascade, the carboxyl group of Gly\(^{76}\) of ubiquitin is activated by ubiquitin activating enzyme (E\(_1\)) (Fig. 1). This step involves the hydrolysis of ATP to PPi to generate a ubiquitinyl adenylate intermediate bound to an E\(_1\) enzyme. Subsequently, an active site Cys residue of E\(_1\) covalently links to ubiquitin via a high-energy thioester linkage, with the concomitant release of AMP. In the second step, following activation, activated ubiquitin is then transferred by transacetylation to a thiol group of an active site Cys residue of ubiquitin-conjugating enzyme (E\(_2\) or Ubiquitin Carrier Protein). Finally, E\(_2\) shuffles ubiquitin either directly to a protein substrate by itself or in cooperation with ubiquitin-protein ligase (E\(_3\)). Thus, the carbon group of Gly\(^{76}\) of ubiquitin is now linked with an \(\varepsilon\)-amino group of protein substrate's internal Lys residue through an amide isopeptide bond. The last step is the transfer of ubiquitin from E\(_2\) to E\(_3\), which accepts ubiquitin in a similar thiol linkage and then to the protein substrate. In some cases, however, covalent linkage between E\(_3\) and ubiquitin is not observed and it appears that ubiquitin is directly transferred from E\(_2\) to protein substrate in a ternary E\(_2\)-E\(_3\)-substrate complex (Glickman and Ciechanover, 2002).

Once the protein substrate is mono-ubiquitinated, a poly-ubiquitin chain is formed through the repetition of the ubiquitination conjugation cascade, in which the carboxyl group of the carboxy terminal of the Gly\(^{76}\) of second ubiquitin is covalently linked to an internal Lys residue of the first ubiquitin (Glickman and Ciechanover, 2002).
The specificity of ubiquitination is largely determined by a series of E3 enzymes and E3 multiprotein complexes, each of which is specific to one or a few corresponding protein substrate(s) and of E2 enzymes, each of which is dedicated to their cognate E3 enzymes. As a result, different combinations of E2 and E3 enzymes allow the selective tagging and degradation of specific intracellular proteins. In contrast, there is a single family of E1, which is highly conserved (McGrath et al., 1991). The deletion of E1 is lethal in yeast and other eukaryotes and the mutation of a putative active site Cys residue abolishes E1 activity (Hatfield and Vierstra, 1992).

2.1.1.2 Proteasomes: the degradation engine

Proteasomes are large multi-subunit proteases that are both free and attached to endoplasmic reticulum and also present in the nucleus of eukaryotic cells (Ye, 2005). Their ubiquitous presence and high abundance in their compartments reflect their central role in cellular protein turnover. Proteasomes recognize, unfold and digest protein substrates that have been tagged by ubiquitin moiety.

The structure of 26S proteasome is well conserved in virtually all organisms from archaeabacteria (Lowe et al., 1995), to yeast (Groll et al., 1997), to humans (Kopp et al., 1997) (Fig. 2). It is composed of two sub-complexes; a 20S core particle that carries the catalytic activity and a regulatory 19S particle that recruits and prepares substrate for degradation. The sub-complex 20S forms a barrel shape core of the complex, to which 19S sub-complexes are attached as caps on both sides of the barrel. However, in some cases, it is present only as a cap on one side of the 20S complex (Goldberg and Rock, 2002). The 20S complex consists of four stacked heptameric rings, forming a central chamber that runs through the stack from top to bottom. Each ring is composed of seven subunits, which may be classified into two groups, i.e., α-subunits that constitute the outer rings and β-subunits that constitute the inner rings, giving 20S complex the general structure of α1-7 β1-7 α1-7 (Ciechanover, 2003). The β-subunit possesses a catalytic site and involves in the cleavage of protein substrates. Both the outer rings are identical to each other. Similarly both inner rings are identical to each other. The exact number of subunits in 19S complex in different organisms may vary. For example, rice 19S particle consists of 21 different subunits (Shibahera et al., 2002), whereas yeast 19S subunit consists of 17 different subunits (Kimura et al., 2003). It is suggested that localization,
Fig. 1: The simple scheme of the ubiquitin-proteasome pathway.

Fig. 2. The 26S proteasomes and its components. An electron tomography image.
cell type and purpose assembly decide the subunit composition of 19S dynamically in the cell environment. The subunits of 19S are generally named as S_n or Rpt_n or Rpn_n.

Although the major role of both ubiquitin and proteasome appears to be in the UPS, both have been suggested to play roles outside this pathway. Ubiquitin has been found attached to histones (Wu et al., 1981) for which, it does not seem to be a degradation signal (Finley et al., 1987). Ubiquitin has also been recognized as a signal for the internalization of plasma membrane proteins (Hicke, 1997), targeting them for degradation in the lysosome/vacuole and it has been reported to act as chaperones in the ribosome biogenesis. The 26S proteasomes are known to degrade some non-ubiquitinylated substrates, as well (Murakami et al., 1992).

2.1.2 HslUV system: prokaryotic homolog of 26S proteasome

The 26S proteasome has archaeal and eubacterial homologs. Most prokaryotes have been shown to have protein degradation machinery homologous to eukaryotic proteasome. Blattner and his coworkers in 1983 identified 26 new heat shock genes in E. coli and termed them as heat shock loci (hsl) genes (Chuang et al., 1993). Of these, HslUV operon has been shown to specify proteins of 19 kDa (HslV) and 50 kDa (HslU). This HslUV complex is shown to mimic eukaryotic 26S proteasome both in structure and function (Fig. 3). The primary sequence of HslV has been shown to be similar to that of certain β-type subunits of the 26S proteasome from eukaryotes and the archaebacterium Thermoplasma acidophilum (Seemuller et al., 1995). In particular, the N terminus of functional HslV protein contains two adjacent Thr residues as in the case of β-type subunits of 26S proteasome, which has been demonstrated to be crucial for its proteolytic activity (Fenteany et al., 1995).

2.1.2.1 Distribution of hslUV genes: throughout prokaryotes and in some protozoans

The hslU and hslV genes are known to be present in all eubacteria other than Actinomycetales. The hslU and hslV genes are present as an operon in E. coli (Bochtler et al., 1997). It is not known if these genes form an operon in the rest of the prokaryotes. A quick text search in ExPAsy retrieves more than 100 hslU and hslV genes and the list of organisms indicates that the genes are present in almost all sequenced prokaryotes. In our lab we have been able to identify HslUV homologs from genomic sequences of different
Fig. 3 Structure of HslUV complex. (PDB id: 1KYI)
parasitic genera like *Plasmodium, Leishmania, Trypanosoma, Cryptosporium* and *Thelaria* (unpublished). In a recent study, the presence of *hslU* and *hslV* genes in primordial eukaryotes *Leishmania* and *Trypanosoma* was reported (Couvreur et al., 2002).

### 2.1.2.2 Primary structure of *hslV* gene

The *hslV* gene in *E. coli* codes for a 19 kDa protein with 170 amino acids. For the protein to be active, the catalytic site residue Thr should be free at the N-terminal with no other preceding amino acids (Yoo et al., 1996). In prokaryotes, generally, the catalytic protease site Thr (T), is situated at the N-terminal, just after the start methionine and N-formyl demethylase is expected to remove the first methionine leaving Thr as the N-terminal amino acid. However, in other organisms, there could be longer stretches of amino acids before the active site. In such cases, a complex processing of protein is required to get a functional protein. Prokaryotic organisms like *B. subtilis, A. aeolicus, T. maritima, C. jejuni, B. burgdorferi* and *R. prowazekii* have more than three amino acids between start methionine and the active site residue at the N-terminal (Couvreur et al., 2002). In *Leishmania infantum* HslV protein was predicted to have a 22 amino acid long signal peptide, which helped in trafficking the protein to mitochondria. Similarly, *T. cruzi* and *T. brucei* have a 19 amino acid long leader sequence (Couvreur et al., 2002) upstream to active site Thr. It is not known, if they are also involved in the trafficking of the HslV protein to the mitochondria. It is also not clear, whether these signal sequences are removed by an autocatalytic protease mechanism.

### 2.1.2.3 Tertiary and quaternary structures of HslV protease

Bochtler and co-workers first crystallized the HslV protein from *E. coli*. The crystal structure of HslV showed that the dodecamer forms a proteolytic chamber (Bochtler et al., 1997). The subunit fold of HslV is the same as in the β subunit of 26S proteasome of *T. acidophilum* (Lowe et al., 1995). HslV differs prominently from archaeabacterial and eukaryotic 26S proteasomes in their subunit assembly. Unlike 20S particle, that are four ring structures displaying 7-fold symmetries, HslV is a dimer of two hexameric rings stacked head to head (Bochtler et al., 1997) (Fig. 4).
Fig. 4: (a) Structure of HslV monomer with ligand LVS bound.
(b) Structure of HslV hexamer (PDB id: 1KYI)
The only major difference between the structures of β subunit of *T. acidophilum* and HslV is due to deletions and insertions in their amino acid sequences. Deletions around helices H1 and H2 and coils S5 and S6 and more importantly the complete absence of strand S10 and helix H5 give, HslV a more compact structure compared to the β subunits of eukaryotic proteasomes. It is already known that at least in *T. acidophilum*, α subunits interact with β subunits that leads to the formation of a heptameric ring, whereas residues important in such an interaction are completely missing in HslUV protein. In the structure of *T. acidophilum*'s 26S proteasome, the peripheral depressions of heptameric rings are filled by their own C-terminal helices of β subunit. This phenomenon is absent in HslV hexameric rings. It was assumed that their absence in HslV protein might lead to tighter packing and the formation of smaller hexameric rings (Bochtler et al., 2000; Song et al., 2000).

2.1.2.4 Primary structure of *hslU* gene

The *hslU* gene in *E. coli* is about 400 amino acid long and codes for a 50 kDa protein having ATPase activity. ATPase domain is present at the N-terminal of the protein. HslU is a member of the Clp / HSP100 family of molecular chaperones (Schirmer et al., 1996). Different functions that have been attributed to the members of this family include facilitation of the degradation of target proteins by cognate proteases as well as the disassembly of oligomeric protein assemblies (Sousa et al., 2000).

Proteins in the Clp/Hsp100 family have either one (HslV and ClpX) or two (ClpA) ATP binding domains. Initial sequence analysis (Neuwald et al., 1999) and the crystallographic structure of *E. coli* HslU protein (Bochtler et al., 2000) unambiguously confirmed that the Clp/Hsp100 proteins are members of the extended AAA+ (for ATPases associated with variety of cellular activities) family (Neuwald et al., 1999). AAA+ family proteins are a group of proteins, whose diverse activities often require the ATP-modulated assembly of oligomeric ring structures (Hanson and Whiteheart, 2005).

2.1.2.5 Tertiary and quaternary structures of HslU ATPase

HslU is a member of HSP100 (Clp) family of ATPases. It is also a member of AAA+ ATPase family (Hanson and Whiteheart, 2005). Crystal structures of HslUV protease from *E. coli* reveal that the folding of individual subunits of HslU fold into three
distinct domains; two globular and the other predominantly α helical (Fig. 5). The first globular domain, N domain, is about 200 amino acids long, contains amino acids from Ser^2 - Leu^32. The second domain; intermediate domain (I domain) is a feature of HslU that is not found in other AAA+ proteins. It is of approximately 130 amino acids and present within the sequence of N domain. It protrudes outward from the N domain at the Met^110 residue and rejoins at Ala^243 residue. The “I” domain is a loosely folded structure with five coiled helices and two β sheets. Carboxy terminal domain (C domain) is formed by residues 333 - 443. It is a four helix bundle with a large bulge at residues Thr^345 - Ser^350 and a double stranded parallel β sheet. The conserved C terminal residues in all HslU family members, FIL (441 - 443), are buried inside the domain with their charges balanced by two Arg residues (Arg^394 and Arg^329) of adjacent HslU subunits (Bochtler et al., 2000).

HslU complex measures 25 nm in length and 12 nm in diameter. Nucleotide is bound at the interface between two subunits. The nucleotide (adenine in ATP/ADP) in HslU subunit is in contact with residues from both the N and C domains. Residues Val^61, Ile^17 and Ile^18 from the N domain and residues Leu^335, Ile^343 and Ala^392 from the C domain form a hydrophobic pocket that accommodates the purine ring of nucleotide. Residues His^16 → Ile^18 and Gly^60 → Val^61 are also involved in nucleotide binding, acting as hydrogen acceptors for the adenine base. Many other residues from N domain, including Lys^63 of Walker A motif interacts with γ phosphate group of the nucleotide. C domain interacts with the β- and γ- phosphates through R393, a residue that is highly conserved among AAA+ ATPases (Schirmer and Lindquist, 1997; Neuwald et al., 1999). Two acidic residues of the adjacent subunit, Glu^286 and Glu^321, are also in contact with the nucleotid. But these residues are not conserved among AAA+ proteins. The absence of the nucleotid seems to lead towards the relaxation of structure, with N and C domains rotating away from each other (Bochtler et al., 2000).

The C domain of one subunit is in contact with the N domain of neighboring subunit in counter clockwise orientation. This contact is so tight that the two domains, which are non-covalently linked, behave as one rigid unit. The movement of N domain is entirely driven by the change in orientation relative to the adjacent C domain.
Fig. 5: (a) Structure of HslU monomer showing N domain (mauve), I domain (pink) and C domain (cyan).
(b) Structure of HslU hexamer with ATP bound. (PDB id: 1KYT)
These domain movements shift the "I" domain and may serve to dissociate sticky substrates and deliver them to HslV. Also during this movement, they lead to a loss of proper six fold symmetry in partially nucleotide bound HslU particle (Bochtler et al., 2000).

Sousa et al. (2000) crystallized the HslUV complex of H. influenzae. In contrast to E. coli structure solved by Bochtler et al. (2000), they found a more compact tertiary structure for HslUV complex (Fig. 3). H. influenzae's HslU and HslV proteins share approximately 80% amino acid sequence identity with their E. coli counterparts. Both models agree well in their subunit organization, subunit fold and hexameric ring structure but disagree in their quaternary structure. Bochtler's model suggests that ATP binding rings are placed distal to the HslV protease, with "I" domains bridging the space between them. In contrast to Bochtler's model, Sousa suggests that the ATP binding region of HslU is in proximity to HslV protease. According to this model, I domain seems to be extended on the poles i.e., distal end from HslV protease. But the position of I domain is not very clear as it is characteristically disordered in all four different crystal structures solved by Bochtler and Sousa.

HslUV from E. coli was also crystallized by Wang (2001). In their model, contact between HslV and HslU is made by nucleotide binding domains, and the pores of HslU and HslV are next to each other and aligned. In the structure proposed by Bochtler et al. (2000), the HslV and HslU pores are separated by more than 80Å. Rohrwild's electron micrograph studies also suggest that most of HslU's scattering mass is adjacent to HslV, with less massive domains extending away from the interface (Rohrwild et al., 1997). The analysis of quaternary structure of HslUV in solution by Sousa, crystal structure by Wang and electron microscopy data by Rohrwild suggests a more compact structure than that of Bochtler's and support Sousa's model for the HslUV complex. Conserved residues have been identified by comparing different HslU sequences. In Wang and Sousa's model, these residues are located in the interface between HslU and HslV proteins. The end of HslU I domain, where HslV binds in Bochtler's model, is not conserved.
Indirect Fourier transforms of experimental scattering curves were computed to get the pair distribution function (P(r)) (a histogram of lengths of all the interatomic vectors of the molecule) for both Bochtler's and Sousa's models. The P(r) fitted well with Sousa's model, though it was not a final proof to discard Bochtler's model. Wang (2001) suggested that crystallographic errors had generated this incorrect structure. He also suggested that once these errors were corrected, a quaternary arrangement that is similar to those observed in the other structures would emerge (Wang, 2001). The orientation of HslU hexamers in the complex suggested a partitioning of activities within the domain of HSIU; the N and C domains may be responsible for the assembly of complex and for the activation of HslV protease; the I domain (HslU specific) may be responsible for selecting substrates for channeling them to the interior of the complex.

In summary, bacterial ATP-dependent protease HslUV has been subjected to EM and crystallographic studies by many workers. These studies have shown that HslV forms a dodecamer of two back to back stacked hexameric rings. HslU forms a hexameric ring and binds to either one or both ends of HslV dodecomer. But the experiments differed, when it came to the quaternary arrangement of HslV and HslU sub-complexes. Three HslUV crystal structures, one small-angle X-ray scattering study, and three EM studies were in good agreement with one another. In these crystal structures, HslU binds to HslV through its nucleotide-binding domains, and HslU residues composing the HslU-HslV interface are conserved. The bound nucleotide in the crystal structure is in an anticonformation. In addition, these structures indicate that the central pores of the peptidase HslV and the ATPase HslU, through which protein substrate is likely to thread from HslU to HslV, are next to each other, aligned and connected. By way of contrast, only one crystal structure indicated a different quaternary arrangement. In this, the nucleotide binding domains of HslU are distal to HslV, and contacts between HslU and HslV are mediated by I domain of HslU.

2.1.3 Biochemical activities of HslUV Proteasome: possesses three peptidase activities

2.1.3.1 HslV protease: cleaves the linear polypeptides presented to it

The only function of HslV (or β subunit in 26S proteasome) is to cleave polypeptides presented to it. Both HslUV and 26S proteasomes contain three different
peptidase activities. (i) chymotrypsin-like activity, (ii) trypsin-like activity and (iii) peptidyl glutamyl peptide hydrolase activity (Lowe et al., 1995). All these three activities are carried out by the same catalytic site, Thr\(^1\) of HslV subunits in HslUV system and β subunits in 26S proteasome. Eukaryotic 20S core particle contains six active sites, three on each of its two β rings, these proteolytic sites differ in their specificities. Two of them, located on β5 subunit, carry out chymotrypsin-like activity; two sites located on β2 subunits, carry out trypsin-like activities. The remaining two active sites located on β1 subunit, carry out peptidyl glutamyl peptide hydrolase activity (Dick et al., 1998; Nussbaum et al., 1998). Substrate binding sites for each of these catalytic β subunits are formed as a result of specific interactions of the catalytic subunit with one of its adjacent β subunits (Lowe et al., 1995; Groll et al., 1997). The 20S proteasome (may be even HslV dodecamer) is not a complex of different individual proteases, but a unique multicalyptic enzyme whose multiple active sites and substrate binding sites form and function only as a complex. Lack of formation of such substrate binding sites in individual β subunits (or in HslV subunit) prevents these proteases from acting on longer polypeptides. These details have not been worked out for HslUV system, yet.

2.1.3.2 Mechanism of peptide cleavage by HslV: similar to that of serine proteases

The mechanism of protein cleavage by HslV is still speculation one and mostly understood from the β subunit of 26S proteasome (Seemuller et al., 1995) (Fig. 6). The unavailability of structures co-crystallized with substrates makes the understanding about the mechanism, a speculation. Most of the understanding about the mechanism came from structures co-crystallized with inhibitors. The first threonine is identified as main catalytic site residue. Threonine (Thr\(^1\)O) is an electro-negative atom that acts as a nucleophile to attack the peptide bond of the substrate (Lowe et al., 1995). This attack results in the formation of the tetrahedral intermediate, which then collapses into an acyl enzyme with the release of the first reaction product. Deacylation of catalytic threonine residues by water molecule leads to the formation of the second product and the regeneration of the free proteasomes. Thr\(^1\)O is in close proximity to Lys\(^33\)N, suggesting a role of accepting protons. It is also suggested that the terminal amino group i.e., the α-amino group of catalytic Thr may accept protons (Groll et al., 1997). Thr\(^1\)N appears to be
hydrogen bonded to Ser\textsuperscript{129} O\textsuperscript{γ}. This serine that is conserved in all HslV proteins of eubacteria and in β subunit of 20S complex in eukaryotes may be necessary to control the protonation of Thr\textsuperscript{1} N, or it may stabilize the orientation of Thr\textsuperscript{1} toward substrate binding pocket. Thr\textsuperscript{2}, in contrast, points away from the inhibitor (Bochtler et al., 1997). Mutational studies show that a mutation Thr\textsuperscript{1} → Ala leads to a reduction of proteolytic activity, whereas, mutation Thr\textsuperscript{2} → Ala abolishes activity, suggesting that the latter substitution results in drastic structural alterations (Missiakas et al., 1996).

2.1.3.3 HslU regulatory subunit: recognizes, unfolds, threads the substrate for cleavage

By itself, HslV has minimal peptidase activity. HslU ATPase stimulates the proteolytic activity of HslV by one to two orders of magnitude. In return, HslV increases the rate of ATP hydrolysis by HslU several fold (Seol et al., 1997). In a HslUV complex, the central pores of HslU and HslV are aligned next to each other but their diameters are so small that only a single polypeptide chain can be threaded through. Therefore, protein substrates with native structure must be unfolded to an extended conformation and translocated into the inner chamber of HslV for proteolysis (Kwon et al., 2004).

The crystal structures of HslU-HslV hexamer have revealed that a large nucleotide-dependent conformational change is transmitted from nucleotide binding pockets to the rest of the structure. Depending on the type and content of bound adenine nucleotides, HslU conformation inter-converts between "open" and "closed" states. This inter-conversion coincides with an up-down movement of the central pore region of HslU relative to bound HslV (Bochtler et al., 2000; Sousa et al., 2000; Wang, 2001; Kwon et al., 2003).

The central translocation pore contains a conserved GYVG sequence that is located next to the Walker box A and the secondary motif is located next to the Walker box B. Tyr\textsuperscript{91} in the GYVG pore motif shows a marked movement from inside of HslU toward inside of HslV, upon the conversion of HslU conformation from open to closed state (Wang, 2001). Park et al. (2005) indeed proved the involvement of GYVG pore motif in the function of HslVU complex through mutational studies. The degradation of natively folded proteins require the flexibility of two Glycine residues (Gly\textsuperscript{90}, Gly\textsuperscript{93}) and
aromatic ring structures of the Tyr⁹¹ amino acid, suggesting that these structural features of the pore motif are essential at least for the step involving the unfolding of native proteins. Further studies using casein (a natively unfolded protein) suggested that they are not essential for the translocation of unfolded proteins (Park et al., 2005). Nevertheless, they are highly preferred. Thus, it can be safely concluded that unfolding and translocation may occur as a coupled process rather than separate steps.

2.1.3.4 Substrate recognition by HslUV: an unknown mechanism

By themselves HslV and ClpP peptidases do not degrade native proteins or even unfolded peptides, whose dimensions preclude diffusion through their entry portals (Sauer et al., 2004). For a protein to be degraded by HslUV protease complex, its polypeptide must be bound, unfolded and translocated through the control channel of HslU ring into the catalytic cavity in the interior of HslV protease dodecamer (Kwon et al., 2004).

In contrast to the related AAA+ proteins, GpX and ClpA and their cognate protease ClpP whose interactions with polypeptide substrates have been studied extensively, there is little information available on substrates for HslUV and the mechanism by which they are selected, unfolded and translocated by HslU to the catalytic cavity of HslV.

In the absence of a universal tag for targets in prokaryotes (like ubiquitin in the case of 26S proteasome), the recognition of specific protein targets for degradation or disassembly may be mediated by a diverse set of unstructured peptide signals displayed on substrate proteins (Sauer et al., 2004). For many AAA+ proteins, substrate recognition is mediated by the binding of unfolders to exposed peptide signals (Gottesman, 2003). It is not clear, however, whether all ATP-dependent proteases use this mechanism of target recognition. It has been suggested, for example, that some energy-dependent proteases simply recognize denatured polypeptides with the length of unfolded segment being a key-binding determinant (Chiba et al., 2000). Some proteins only become substrates for degradation by AAA+ proteases following prior cleavage by another protease.
In some cases, adaptor proteins may be required for efficient substrate degradation or disassembly by AAA+ enzymes. Adaptors can also inhibit the recognition of specific substrates (Sauer et al., 2004).

HslUV has a substrate specificity overlapping that of Lon protease, another ATP-dependent protease in which a single subunit contains both proteolytic active site and ATPase domain. Lon mutants are UV sensitive due to the stabilization of SulA. Lon mutants are also mucoid, due to the stabilization of another Lon substrate, RCSA (positive regulator of capsule transcription). The over production of HslUV suppresses both of these phenotypes. This suggests that the substrate specificities of Lon and HslUV proteases are overlapping. It has been proposed that, HslUV acts as a back up for Lon under certain conditions like elevated temperature (Wu et al., 1999). Extensive experiments by Kanemori and co-workers suggested that HslUV degrades cell division inhibitor SulA *E. coli*, at least *in vivo* (Kanemori et al., 1999).

The $\sigma^{32}$ is another protein that is found to be degraded by HslUV system. $\sigma^{32}$ is a transcription factor, synthesized in large amounts during heat shock, to induce the expression of heat inducible genes. Studies by Kanemori et al. (1999), showed that a set of ATP-dependent proteases appear to play significant roles in the negative control of heat shock response, by modulating *in vivo* turnover of $\sigma^{32}$ as well as through the degradation of abnormal proteins. Further studies with the deletion mutation of HslUV and other proteases revealed that along with HslUV, other proteases also play a role in controlling the amount of $\sigma^{32}$ (Kanemori et al., 1999).

In a set of elegant experiments Saucer and co-workers had showed that Phage P22-Arc repressor is a target protein for HslUV (Burton et al., 2005). Both wild Arc protein and Arc protein fused with a mechanically and highly stable protein are degraded equally well by HslUV system. By fusing different regions of Arc with another protein, it is established that sequences located within residues 1-13 of native Arc are sufficient to direct it for degradation by HslUV, whereas, signals within 14-24 enhance its binding to HslUV and leads to efficient degradation. HslUV system was not able to degrade the ARC protein when seven amino acids at the N-terminal of the Arc protein are truncated (Burton et al., 2005).
To probe the specificity of peptide binding and to identify sequence changes that might improve HslU binding, peptides with single-residue substitutions of Arc 1-13 sequence were prepared and checked for their binding to HslU protein. Based on the binding affinities of different peptides, a 12-residues peptide (NH₂-MRYFFKKKLKY-COOH) was designed and synthesized. This peptide showed greater binding to HslU than native Arc sequence (Burton et al., 2005).

Binding and hydrolysis of ATP are critical in powering the HslUV degradation of protein substrates, but it is not clear whether the bound nucleotide also plays a role in substrate recognition. Burton et al. used ARC 1-13 peptide to test the effect of nucleotide on substrate binding. Strong binding was observed with ATP⁵⁻Mg²⁺ and ATP-Mg²⁺ but not with ATPγ₅, ADP. These results suggested that the conformation of HslU in the presence of nucleotides favors substrate binding. It has been proved that peptides bind HslU and inhibit the degradation of specific substrates (Burton et al., 2005).

2.2 Protozoan parasites and HslUV system: employs both prokaryotic (HslUV) and eukaryotic system (26S)

Until 2000, HslUV proteasomes were considered to be present only in eubacteria. Our lab is the first to identify the presence of this prokaryotic gene in the genomic sequence of P. falciparum genome (unpublished data). Also ORFs identical to hslV genes were also found in unfinished genomic sequences of other Plasmodium sp like P. vivax and P. yoelii and in Leishmania, while all these organisms also contain 26S proteasome machinery (unpublished data). Later in 2002, Couvreur and co-workers identified hslV gene from a number of protozoan parasites. The presence of HslUV system and 26S proteasome was considered mutually exclusive (Couvreur et al., 2002).

2.3 Proteasome inhibitors: reagents for basic study and drug development

Much of the initial understanding about the importance of this regulated protein degradation pathway in the regulation of different cellular processes came from biochemical studies in extracts of mammalian cells and genetic studies in yeast (Ciechanover and Schwartz, 1994). However, knowledge about its physiological roles in mammalian cells was slow to develop until cell-permeable proteasome inhibitors were developed, which greatly simplified such studies. A number of proteasome inhibitors,
both peptide and non-peptide inhibitors, have been developed by different workers. Although, the proteasome has multiple active sites, inhibition of any one of them usually hinders the overall protein degradation.

2.3.1 Peptide inhibitors: peptide aldehydes and peptide boronates

2.3.1.1 Peptide aldehydes: first ones to identify and widely used

Peptide aldehydes were the first proteasome inhibitors to be developed and are still the widely used inhibitors (Fig. 8). Early studies indicated that certain activities of proteasome are inhibited by specific peptide aldehydes that are substrate analogs (Vinitsky et al., 1992; Rock et al., 1994). For example, chymostatin and acetyl-leu-leu-norleucinal (MG101, also called calpain inhibitor 1) reversibly inhibit chymotrypsin-like activity, whereas leupeptin inhibits trypsin-like activity. A systematic effort to synthesize more potent proteasome inhibitors was undertaken at ProScript, Inc. (formerly Myogenics, Inc.), Cambridge, Massachusetts. One such agent, CBZ-leu-leu-leucinal (MG132), is a potent inhibitor of chymotryptic activities and it readily enters cells. This agent blocks protein breakdown in mammalian cells and in permeable strains of yeast and has been made available for investigative purposes. A similar but less potent inhibitor is CBZ-leu-leu-norvalinal (MG115). Both aldehydes reduce the degradation of Ub-conjugated proteins by the 26S complex without affecting its ATPase or isopeptidase activities. Interestingly, their potency against activated 20S particle is greater than against 26S proteasome. These peptide aldehydes block up to 90% of proteolysis in cultured mammalian cells, including the degradation of highly abnormal proteins, and short- and long-lived normal proteins, which represent the bulk of cell proteins. Thus, the proteasome is the primary site for breakdown of most proteins in growing cells, although lysosomal proteolysis can be important in non-growing cultures and in specialized cells. These inhibitors or other peptide aldehydes have been used to implicate the proteasome in the rapid degradation of many short-lived proteins, such as IκB-α, in MHC-class I antigen presentation, in the limited processing of NF-κB and in the breakdown of membrane proteins including CFTR on the endoplasmic reticulum and transmembrane tyrosine kinase receptors (Craiu et al., 1997; Craiu et al., 1997a).
Several features of MG132 and 115 are of particular advantage for research purposes; (a) their effects are reversible and, after removal, the normal rates of protein degradation are restored within 30-60 min; (b) their efficacy in reducing proteolysis in intact cells appears greater than in crude extracts or with purified 26S proteasome particles and (c) cells are fully viable for at least 10-20 h in their presence, and during this time the rates of protein synthesis and ATP content are unaltered. With time, ubiquitinated proteins accumulate and cells express heat-shock genes, a classic stress response and, as a consequence, such inhibitors can induce peptide aldehydes and also affect lysosomal and Ca\(^{2+}\)-activated proteases. It is important in such studies to also show that the selective inhibitors of lysosomal proteolysis (e.g. weak bases) or of calpains (e.g. E64) do not have similar effects, and/or that the sensitivity of a response to different aldehyde inhibitors correlates with their potency against proteasome (Kisselev and Goldberg, 2001).

Several other proteasome inhibitors have been reported, but have not been studied as extensively. Wilk and co-workers introduced a peptide aldehyde CBZ-Ile-Glu(O-t-Bu)-ala-leucinal, which can also enter cells, reduce the breakdown of 1kB and cause the accumulation of Ub-conjugates (Figueiredo-Pereira et al., 1994). Siman and co-workers have synthesized a number of potent dipeptide inhibitors that contain a hydrophobic chain on the α-amino terminus; they inhibit the chymotryptic activity, reduce antigen presentation on MHC-class I molecules and stabilize short-lived mutant proteins (Harding et al., 1995). A novel group of potent peptide aldehydes has been synthesized recently that contains large hydrophobic groups at the P4 position, which cause a dramatic increase in potency. Finally, Adams and coworkers have synthesized very potent, new non-aldehyde inhibitors that block multiple activities of 20S particles and 26S proteasome, reversibly and inhibit ubiquitin-dependent proteolysis in vivo (Adams, 2001).

2.3.1.2 Peptide boronates: more potent, selective inhibitors

Peptide boronates are much more potent inhibitors of the proteasome than the aldehydes (Fig. 8). The mechanism of inhibition by these slow binding compounds is yet to be confirmed by X-ray diffraction, but assumed to act like peptide aldehydes. Boronate inhibitors are more selective than that of peptide aldehydes. Boronates, unlike aldehydes,
are not inactivated by oxidation and are not rapidly secreted from cells by multi-drug resistance genes (Kisselev and Goldberg, 2001). This combination of potency, selectivity and metabolic stability makes the peptide boronates, better drug candidates. One of the dipeptide boronates, PS-341, is currently in phaseII clinical trials in cancer patients (Adams, 2001).

2.3.2 Non-peptide inhibitors: lactacystin and \( \beta \)-lactone

Lactacystin, a microbial (Streptomyces) natural product that inhibits cell proliferation and induces neurite outgrowth in a murine neuroblastoma cell line, has become a widely used reagent (inhibitor) in the functional studies of both prokaryote and eukaryote proteasomes (Omura et al., 1991) (Fig. 8).

Lactacystin binds certain catalytic subunits of HslV / \( \beta \)-subunit of 26S proteasome and inhibits the three best-characterized peptidase activities of proteasomes, but at different rates. Two of the activities are inhibited irreversibly and one reversibly (Craiu et al., 1997). It modifies the side chain of active site residue Thr and thereby irreversibly inactivates it (Fenteany et al., 1995).

Reactive nucleophile (from the side chain amino group of Thr) attacks the C4 group of inhibitor, which leads to the acylation of active site. The acylation of target by lactacystin was envisioned to occur possibly through the formation of clasto-lactacystin \( \beta \)-lactone as an active intermediate, resulting in the cyclisation of lactacystin. Clasto-lactacystin is covalently attached to its target (Kisselev and Goldberg, 2001). The conversion of lactacystin into clasto-lactacystin via an intermediate \( \beta \)-lactone is a spontaneous process (Fig. 7).

Lactacystin inhibits chymotrypsin-like and trypsin-like activities irreversibly but at different rates. \( \beta \)-lactone inhibits each of the three activities 15-20 times faster than lactacystin, with the same order of effectiveness (Fenteany et al., 1995). Currently, this is the only compound known to inhibit proteasome specifically, without inhibiting any other proteases; also it does not inhibit lysosomal protein degradation in the cell (Goldberg and Rock, 2002). This is in contrast to other commonly used proteasome inhibitors such as peptide aldehydes and 3,4-dichloro iso-coumarin, which inhibit a wide range of proteases. Treatment with the lactacystin blocks the morphological changes in
Fig. 6. Proteasome catalytic mechanism. Substrate is black, proteasome is cyan, and bonds formed during catalysis are blue.

Fig. 7: Mechanism of proteasome inhibition by lactacystin and β-lactone
Fig. 8: Major proteasome inhibitors. Pharmacophores are in red.
Trypanosoma cruzi and affects the development of both liver and intra-erythrocyte stages of Plasmodium spp (Gonzalez et al., 1996; Gantt et al., 1998).

2.3.3 Peptide vinyl sulfones

Peptide vinyl sulfones are synthetic irreversible inhibitors of proteasome (Bogyo et al., 1997). They covalently modify Thr1 of both HslV and β subunit. Two compounds most widely used as probes are NLVS, which modifies predominantly the chymotrypsin-like subunits, and IYL3VS, which reacts with all subunits.

2.3.4 Epoxyketones

Recently discovered natural epoxyketones (epoxomycin & eponemycin) exert their anti-tumor activities by inhibiting the proteasomes. Epoxomycin reacts primarily with chymotrypsin-like active site. Studies on yeast proteasomes co-crystallized with epoxomycin revealed that the catalytic hydroxyl first attacks the carbonyl group of the pharmacophore. Then the free α-amino group of the threonine opens up the epoxy ring and completes the formation of the morpholino adduct (Groll et al., 2000). Epoxyketones, because of their unique mechanism, are the most selective inhibitors of the proteasome known (Meng et al., 1999; Meng et al., 1999a).

The chymotrypsin-like activity of the proteasome can also be inhibited by several major drugs, which were all developed as inhibitors of other enzymes. They are ritonavir, an inhibitor of the HIV-encoded aspartic protease (Schmidtke et al., 1999), the lactonized pro-dug form of the HMG-CoA reductase inhibitor lovastatin (Rao et al., 1999), the anticancer DNA-intercalating agent aclacinomicin A (Figueiredo-Pereira et al., 1996) and the immuno-suppressive agent cyclosporin A (Meyer et al., 1997). Mechanism of action of these drugs on proteasome is not known yet.

2.4 Proteasome inhibitors as antimalarials

Experiments by many scientists (Gonzalez et al., 1996; Gonzalez et al., 1997; Gantt et al., 1998) revealed that the proteasome inhibitors indeed affected the growth of Plasmodium falciparum both in vivo and in vitro, and in both liver and intra-erythrocytic stages. But, with the identification of HslUV system in Plasmodium, it is now not clear whether 26S proteasomes are solely responsible for the observed effects.
Treatment with lactacystin blocks the morphological changes of *Trypanosoma cruzi* at two separate stages of its life cycle. The transformations of trypamastigotes into amastigote-like organisms in axenic medium, and the intracellular development of the parasite from amastigotes into trypomastigotes, are prevented by lactacystin or peptide aldehydes that inhibit proteasome function (Gonzalez et al., 1996).

Lactacystin blocks the development of pre-erythrocytic and intra-erythrocytic stages of *Plasmodium* spp. Lactacystin treated sporozoites, although invasive *in vitro*, did not round up normally. This arrest of the development of exo-erythrocyte form morphology was supported by no increase in A-type rRNA even 20 h after invasion. A-type rRNA (a major molecular marker of the developed stage of sporozoites) is the major type of rRNA seen 20 h after the invasion of hepatocytes by normal sporozoites. Similarly, the development of sporozoites *in vivo* also appeared to be inhibited by lactacystin, as treated sporozoites were at least 10 fold less infective in mice (Gantt et al., 1998).

During the intra-erythrocytic part of life cycle, lactacystin-treated trophozoites, however, do not transform into schizonts. They maintain an arrested but apparently normal morphology for extended periods. The addition of lactacystin up to 30 h after erythrocyte infection strongly inhibited \(^{3}H\)hypoxanthine incorporation, but the drug had no effect after the beginning of schizogony. Although lactacystin-treated schizonts appeared normal by light microscopy and incorporated \(^{3}H\)hypoxanthine normally, they did not rupture. An attractive possibility is that the drug affects the control of cell cycle progression in the parasite (Gantt et al., 1998).

26S proteasome activity is required for transition through G1/S boundary and for exit from M phase in a number of cell types (King et al., 1996). HslUV complex is also implicated in the regulation of cell division in *E. coli* through regulating cell division inhibitor SulA (Kuo et al., 2004). Cell cycle control in *Plasmodium* is poorly understood. Both HslUV and 26S proteasomes in *Plasmodium* may play similar roles.

2.5 History of malaria

Malaria has been a major cause of human suffering for thousands of years and despite considerable advances in our understanding of the disease, it continues to be one
of the main causes of serious illness and death in the world. It is caused by a protozoan parasite belonging to the genus *Plasmodium*. Human malaria is as old as mankind. Hippocrates in 400 B.C. gave first clinical description of malarial fever. In the middle of the 17th century, malaria was treated by giving the extract made from the bark of Peruvian tree, now known as chinchona tree. In the year 1820, Pelletier and Cavention, isolated an active ingredient from the bark of chichona and named it quinine, which was found to have an anti-malarial effect. In 1880, Lavern, a French army surgeon in Algeria, first observed and described malarial parasites in the red blood cells of man. Sir Ronald Ross, in 1887 described sporogony of *Plasmodium relictum* in *Anopheles* mosquitoes and also showed that *P. falciparum* was transmitted to humans through the bite of mosquitoes.

To date, four species of *Plasmodium* are known to infect humans, namely, *P. vivax*, *P. falciparum*, *P. ovale* and *P. malariae*. These four species differ morphologically, immunologically, in geographical distribution, relapse pattern and drug response. Of the four human malaria species, *P. falciparum* causes hemorrhagic malaria, which is fatal. In India, *P. vivax* and *P. falciparum* are widely prevalent. WHO launched malaria eradication programme during 1955-56 in many countries, which achieved unprecedented success. However, in some areas the disease re-emerged mainly due to two factors, 1) the development of multiple drug resistance in parasite and 2) the development of resistance in mosquito vectors against many insecticides. Therefore, new tools and improved use of control measures are essential for effective control of malaria.

### 2.6 Life cycle of malaria parasite

*Plasmodium*, the causative agent of malaria completes its life cycle (Fig. 9) in two hosts: human and mosquito. In human, it undergoes asexual cycle and in mosquito it goes through sexual cycle called sporogony.

#### 2.6.1 Life cycle in humans

Malaria infection is initiated when an infected female *Anopheles* mosquito injects sporozoites into the vertebrate host while taking a blood meal. Sporozoites, which disappear from the blood in less than an hour, invade host hepatocytes (Fig. 9). The mechanism of hepatocyte invasion of sporozoites is poorly understood, although some of
The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host 1. Sporozoites infect liver cells 2 and mature into schizonts 3, which rupture and release merozoites 4. (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells 5. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites 6. Some parasites differentiate into sexual erythrocytic stages (gametocytes) 7. Blood stage parasites are responsible for the clinical manifestations of the disease.

The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal 8. The parasites' multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's stomach, the microgametocytes penetrate the macrogametocytes generating zygotes 9. The zygotes in turn become motile and elongated (ookinetes) 10, which invade the midgut wall of the mosquito where they develop into oocysts 11. The oocysts grow, rupture, and release sporozoites 12, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites 1 into a new human host perpetuates the malaria life cycle.

Fig. 9.: Life cycle of malaria parasite.
the reports have shown the involvement of kupper cells in hepatocyte invasion (Yoshida et al., 1980). Two major proteins, circumsporozoite protein (CSP) and thrombospondin related adhesive protein (TRAP), on the surface of sporozoite were shown to be responsible for invasion process. Once in the hepatocyte, sporozoites over a period of 2-10 days (for *P. falciparum*) mature to liver-stage schizonts, which rupture releasing up to 40,000 merozoites. These merozoites then escape into the sinusoids of liver and invade erythrocytes.

2.6.1.1 Erythrocytic cycle

Merozoites released at the end of exo-erythrocytic cycle are ovoid or elongated structures and vary in size. Merozoites proceed to invade erythrocytes (Mitchell and Bannister, 1988) and this process has been studied extensively through electron microscopy (Hadley, 1986). Erythrocyte invasion by merozoite involves an initial attachment at any point on the erythrocyte and subsequently reorient in a way that the apical end of the parasite comes in contact with RBC membrane. This contact induces the release of microneme proteins, which subsequently lead to the formation of "Tight Junction" (Aikawa et al., 1981). Meanwhile, the contents of apical organelles are released around erythrocyte membrane and vacuole is formed (Bannister et al., 1977). As invasion proceeds, the vacuole inside the merozoite leads to a 'ring' stage.

The vacuole surrounding the parasite is called parasitophorous vacuole. Parasite ingests hemoglobin, which is digested into hemozoin, the typical malaria pigment. At this stage, parasite is termed as trophozoite. At this time, infected erythrocytes become distorted and nuclear division starts. Cytoplasm surrounds each nucleus and a schizont containing several merozoites is formed. The mature schizont ruptures releasing 10-30 merozoites, each of which can reinvade another erythrocyte. Alternatively, merozoites can develop within the erythrocyte to give sexual forms called macrogametocyte (female gametes) and microgametocyte (male gametes).

2.6.2 Sporogony in mosquito

When a mosquito ingests erythrocytes containing gametocytes, the gametocytes transform into gametes. Gametes are released from the erythrocytes in the midgut of insect, followed by fertilization leading to 'zygote' formation within 18 h of blood meal.
This immobile zygote elongates into motile ookinite, which enters into intestinal epithelium and comes to rest beneath the basal lamina forming an oocyte 24-72 h after blood meal. Oocyte matures in 7-15 days after the blood meal, gives rise to as many as 10,000 sporozoites, which escape through pores in the membrane of oocyte and travel into haemocytic fluid to accumulate in the acinal cells of salivary gland. Here, sporozoites mature into infective sporozoites that are inoculated into a vertebrate host as mosquito takes its blood meal.

2.7 Current situation of malaria

Malaria still remains a major parasitic disease, in spite of more than a century of efforts to eradicate it. About 300-500 millions people come in contact with malaria every year and around 1.5 million people succumbed due to the disease. Thus, malaria is one of the major infectious diseases and it exerts an enormous toll on developing countries throughout the tropics. In particular, cerebral malaria is an overwhelming problem in sub­saharan Africa, where the most malarial morbidity and mortality occur. Taking these into consideration, international agencies have recently initiated new programs that will provide increased funds for malaria control and research. The roll back malaria initiative was formed in 1998 to expedite and coordinate worldwide malaria control efforts. The principle focus of these programs is to address the pressing needs of those, who are at risk of severe malaria particular by children and pregnant women in Africa and other endemic regions. In addition, two important programs were established in 1999: malaria vaccine initiative and medicine for malaria venture. Importantly, these programs are much better funded than the other existing national and international programs for malaria control. In spite of huge funding and major advancements in the field of vaccine and drug, malaria still remains a major health problem in developing countries. This drawback is because of the poor understanding of the biology of the parasite.

An international effort was launched in 1996 to sequence the genome of Plasmodium falciparum with the expectation that the genome sequence would open new avenues for research. The complete genome sequence of P. falciparum was published in the year 2002 (Gardner et al., 2002). The annotation of completed P. falciparum sequence revealed that of the 5,334 predicted proteins; about 60% are hypothetical, which means that these proteins do not have sufficient similarity to proteins in other organisms
to justify the provision of functional assignments. Hence, it is very much essential to
develop tools to study the functions of these genes. For the same, in recent years,
transfection technology has been developed in *P. falciparum*, *P. berghei*, *P. knowlesi* and
*P. yeolii*.

In this present study, we identified and biochemically characterized genes that
counted HslUV system from *P. falciparum*. Also, we studied the indispensability of
the HslUV system for the survival of parasite using RNAi technique against *P.
falciparum* *hslV* gene.