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Tanveer Ali Dar
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Proteostasis and Chaperone Surveillance

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Preface

Human diseases have been the matter of gravest concern ever since the conception of scientific research. Whether it is basic or applied research, all intend to cater one single curiosity of man, and that is attainment of illness-free healthy life. Indeed, we are in an era where many of the earlier deadly considered disorders no longer exist, but still we have to cover a very long distance in order to find cure for all. However, in this search we have realized that it is the basic biomolecules that are of esteem importance and has a great deal of involvement in disease development. Protein is one such biomolecule of keen interest for researchers as it is responsible for the ultimate biological function. It is the fine balance of proteostasis inside the cell that is responsible for smooth functioning of various cellular activities, and any lapse in this molecular balance can lead to a fatal disorder. For instance, loss of function of a protein either due to impaired protein synthesis or improper protein folding is known to cause various disorders such as cystic fibrosis, phenylketonuria, etc., and on the other hand, gain of function can generate a cohort of aggregated species leading to various incurable disorders like Parkinson's and Alzheimer's disease. To ensure proper management of proteostasis, including protein synthesis, its proper folding, and transportation to the correct target region, cell has evolved an effective surveillance system. Innumerable research carried out to date has clearly indicated the molecular and chemical chaperones to be an essential part of this surveillance system. They not just ensure proper folding and targeting of the protein to respective organelles but also remove the improperly folded protein and various aggregates of proteins by targeting them toward the cellular debris clearance system, including proteasome, lysosome, and cell-mediated autophagy. Moreover, there are various reports which show failure in chaperone surveillance to be the reason behind the aggressive disease progression. Therefore, in the hope of finding the novel ways of curing proteopathies by altering these modulators and sentinels of protein homeostasis, it is important to understand the role of chaperone surveillance in maintaining the fine molecular balance of cellular proteostasis. This edited volume is made with an intention to help a curious beginner as well as an expert who sought the knowledge regarding protein homeostasis, the diseases that could develop due to imbalance of this homeostasis, and complete erudition of the current assessment of molecular and chemical chaperone surveillance.

The edited book entitled *Proteostasis and Chaperone Surveillance* comprises 9 different chapters arranged in three different sections that enable the

book to cover and highlight major aspects of proteostasis and chaperone functions. Section I is introduced to give a complete picture of maintaining proteome or protein stability in the cells. The section allows one to understand that there are different approaches of maintaining protein stability via structural allostery (Chap. 1) that we explained with a suitable transcription factor adaptor protein, protein posttranslational modifications (Chap. 2), and small molecule chaperones (Chap. 3) that we discuss with several small molecules and enzyme systems. Section II basically deals with the consequences brought about due to the failure of the proteostatic system. Each of the different chapters under (Chaps. 5, 6, 7, and 8) this section describes the different proteo-pathologic conditions making the section covered with almost all proteopathic mechanisms. Section III is dedicated about the chaperonic machineries that cells have, to cope up with the changing need of the proteostatic system. This section gives a detail information of the almost all of the treatment strategies of diseases caused due to failure of the proteostatic system. Chapter 9 under this section also covers the pharmaceutical strategy of inhibiting proteostatic failure by using small molecule compounds.

Chapters contributed in this book have been published keeping intact authors' justifications. Necessary editorial changes were made wherever required, and authors have been requested to revise the manuscripts multiple times to address the editorial issues. We tried our best to gather information on different aspects of this volume; however, there is a possibility of some errors still creeping in the book for which we seek reader's indulgence and feedback. We owe our sincere thanks to the authors for their valuable contribution. We are also very thankful to Springer India Limited and their staff members who were directly or indirectly associated with this project for their constant help, valuable suggestions, and efforts in bringing out the timely publication of this volume.

Delhi, India
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Part I

Maintaining Proteostasis

Structural Allostery and Protein–Protein Interactions of Sin3

1

Tauheed Hasan and Daman Saluja

Contents

Abstract

Sin3, a global transcription regulator, acts as a molecular scaffold for complex assembly and also as a molecular adapter bridging HDAC (histone deacetylase complex) with an astonishingly large and diverse group of DNA-binding transcription factors and chromatin-binding proteins. Most of the protein-protein interactions are achieved through six conserved domains of Sin3 that include four paired amphipathic helices (PAH 1–4), one histone deacetylase interaction domain (HID) and one highly conserved region (HCR). These PAH domains, though, are structurally homologous to one another having different amino acid sequences which result in differential protein-protein interactions. The PAH domains also show conformational flexibility under different physiological conditions such as pH and temperature and conformational heterogeneity upon interaction with different proteins. Interaction with large groups of transcription factors and co-repressor complexes is also possible due to the fact that Sin3 shows structural modification such as phosphorylation, myristoylation, ubiquitination, and SUMOylation as well as structural allostery

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upon interaction with proteins. Till now, only N-terminal region of Sin3 has been characterized, which makes it more important to characterize C-terminal region of Sin3 so as to find and understand its interacting partners and their role in Sin3-mediated gene regulation. Future studies should also be directed towards understanding the regulation of Sin3 protein under different physiological conditions and modulation of its biological activity.

Keywords

Sin3 complex • Protein-protein interaction • Allosteric regulation • Post-translation modification • Co-repressor • Ubiquitination

1.1 Introduction

Sin3, a global transcription regulator, helps to regulate many biological functions including nucleosome remodelling, DNA methylation, cell proliferation and apoptosis. Sin3 does not bind to DNA but is a scaffold protein that helps the transcription of various genes by interacting with different transcription factors, forming Sin3 complex (Grzenda et al. 2009). The core complex of Sin3 consists of eight components in humans: Sin3, SAP18, SAP30, HDAC1, HDAC2, RbAp46, RbAp48 and SDS3 (McDonel et al. 2009). Presence of different sub-complexes has also been reported in different organisms; these sub-complexes contain additional components besides the components present in a core complex. Sin3 interacts with numerous transcriptional factors through its six distinct yet conserved domains that include the four imperfect repeats of paired amphipathic helices (PAH 1–4), histone deacetylase interaction domain (HID) and highly conserved region (HCR) (Grzenda et al. 2009). These PAH domains, though distinct in sequence, are structurally homologous to one another and recognize unique sets of proteins. NMR and X-ray crystallographic studies of individual PAH domains as well as PAH domains complexed with DNA-binding domains and co-repressor complex revealed that each PAH domain folds in a distinct way to interact with the proteins. There are a number of other factors which are responsible for making PAH domains an ideal region of Sin3 for interacting with large numbers of transcription factors. Presence of different splice variants and isoforms of Sin3 in humans and various other organisms further increases the flexibility of Sin3 protein to recruit a large set of proteins (Ayer et al. 1995). Interaction of Sin3 with the proteins indirectly via co-repressor complex adds another level of complexity to Sin3 functions. Post-translation modifications of Sin3 such as phosphorylation, myristoylation, ubiquitination and SUMOylation help in maintaining protein-protein stability and proper regulation of genes. Structural variation in domains of Sin3 and allosteric regulation of Sin3 further increase the flexibility and conformational heterogeneity of Sin3 protein, making it an ideal regulator of gene expression.

1.2 Sin3 Complex

Sin3 is a large protein which is thought to function as a molecular scaffold due to presence of several protein-protein interaction domains. It helps in the assembly of co-repressor complex and as a molecular adapter to bridge components of the complex with DNA-bound repressors (Sheeba et al. 2007; Silverstein and Ekwall 2005). Chromatographic data of components of Sin3 complex suggested that there are different Sin3 complexes and these are variable in their structural components. Certain proteins are found to be conserved between them and thus referred to as "core complex" while some have additional proteins. The mammalian Sin3 complex analogous to Sin3/RPD3 complex of yeast comprises of at least seven polypeptides besides Sin3, including HDACs HDAC1 and HDAC2, two histone-binding proteins RbAp46 and RbAp48 (Rb-associated polypeptides) and three Sin3-associated polypeptides SAP18, SAP30 and SAP45/Sds3. Human family with sequence similarity 60 member A (FAM60A) protein, a new member of Sin3 core complex, has been discovered recently (Smith et al. 2012). Other protein components might vary between different sub-complexes. $p33^{ING1b}$ is one such example as it is thought to associate with only certain species of

Sin3/HDAC complex (Campos et al. 2004). Similarly, Sin3A, an isoform of Sin3 present in mammals, contains five extra polypeptides besides components of core complex which includes three SAP polypeptides SAP25, SAP130, SAP180 and one RBP1 (Rb-binding protein 1) protein (Xie et al. 2011). In the years following the elucidation of the core complex, a number of other associated proteins were uncovered, including BRMS1, CpG methylated binding protein (MeCP2) and ING1/2 (Grzenda et al. 2009; Nan et al. 1998; Zhang et al. 2014). The ability to support different compositions within the complex may be yet another way to expand the functional flexibility of Sin3 complex.

In the core complex, Sin3 is the platform for protein interaction, most importantly the enzymatic activity of HDAC1 and HDAC2. RbAp46 and RbAp48 are able to bind histone H4 and H2A and might thus function in stabilizing the interaction between co-repressor complex and chromatin. HDAC1 and HDAC2 bind to conserved HID region on Sin3 and provide the enzymatic activity to the complex. RbAp46 and RbAp48 that bind to nucleosomal histones are involved in chromatin modelling such as histone acetylation, nucleosome remodelling and nucleosomal assembly (Spencer and Davie 1999). Sin3-associated proteins (SAP) provide structural support and stabilize the complex. Brief functions of different components of Sin3 complex present in different organisms are listed in Table 1.1.

1.3 Structural Overview

The basic structure of Sin3 is evolutionary conserved from yeast to mammals. It contains four paired α-helices known as PAH (paired amphipathic helix) domains separated by less conserved amino acids forming the spacer region. There are also two other conserved protein interaction domains, HID (histone interacting domain) located between PAH3 and PAH4 regions and HCR (highly conserved region) situated C-terminally to PAH4 (Fig. 1.1). The PAH domains are meant for protein-protein interaction; HID interacts with HDACs and many other components of Sin3/HDAC complex and HCR, recently identified as another protein interacting domain (Grzenda et al. 2009). The current evidence advocates Sin3 as a modular protein where PAH1-3 are earmarked for interactions with different transcription factors while the HID and PAH4 domains primarily serve scaffolding function by interacting with other components/subunits of the repressor complex. PAH1-3 domains form pre-folded binding modules on full-length Sin3, like a beads-on-a-string model (Le Guezennec et al. 2006). Unlike the other PAH domains, PAH4 most likely does not fold as a four-helix bundle but instead adapts a distinct fold (van Ingen et al. 2006). Out of the four Sin3 PAH domains, the tertiary structures of only mSin3A and mSin3B PAH1 and PAH2 domains have been determined by NMR and X-ray crystallography (Kumar et al. 2011; Nomura et al. 2005; Sahu et al. 2008). The fundamental structures of both PAH1 and PAH2 are similar; however, the helices formed in PAH1 are shorter than that of PAH2 (Sahu et al. 2008). The Sin3A PAH2 domain homodimerizes and exists in unfolded state, but Sin3B PAH2 domain is monomeric and is fully folded while PAH1 exists in homodimeric form in both isoforms. Thus, PAH2 domain shows conformational heterogeneity in two isoforms of Sin3 (Kumar et al. 2011). Structures of PAH1, PAH2 and PAH3 complex with SID domain of interacting partners have also been determined by NMR and X-ray crystallography (Sahu et al. 2008; Swanson et al. 2004). PAH2 domains of mSin3A and mSin3B hold a wedged four-helix bundle structure associated with the Sin3-interacting domain (SID) of Mad1, a transcription factor involved in cell proliferation and differentiation in mammalian cells. PAH2 domain complex with the Mad1 SID adopts an amphipathic α- helix whereas PAH1 holds a rather globular four-helix bundle structure with a semi-ordered C-terminal tail on interaction with the NRSF/REST repressor domain (Nomura et al. 2005). Solution structure of PAH3 and SAP30 complex showed that PAH3 forms a canonical hydrophobic cleft and a discrete surface formed largely by the PAH3 α2, α3 and α3′ helices. Sin3 interaction domain (SID) of SAP30 binds to PAH3 via a tripartite structural motif, including a C-terminal helix that targets the

Table 1.1 Components present in Sin3 core complex in different organisms

Component of Sin3 complex	Functions	References
Sin3	Act as a molecular scaffold to provide a platform for the assembly of numerous transcription factors and co-repressor complex and also as a molecular adapter bridging components of the complex with DNA-bound repressors	Binda et al. (2006)
HDAC1/2 (Histone deacetylase complex)	Constitute the major catalytic subunits of Sin3/HDAC complexes and provide enzymatic activity resulting in deacetylation of both histones H3 and H4	He et al. (2009)
RbAp46/48 (Retinoblastoma-associated proteins)	Rb-associated proteins can interact with histone H4 and H2A and thus are predicted to help stabilize the interaction between the Sin3/HDAC complex and histone H4 and target the Sin3/HDAC to nucleosome and thus stabilize the interaction of core-complex with chromatin	Grzenda et al. (2009)
FAM60A	Newest member of Sin3 core complex. Interact with HDAC1/2 and stabilize the core complex. It also helps in repressor of TGF-beta signalling and cell migration	Smith et al. (2012)
SAP18	Ubiquitously expressed in all mouse tissues tested and interacts directly with both mammalian Sin3 and HDAC1. Besides stabilization function of the Sin3 core complex it also has been discovered to interact with GAGA factor in drosophila and Chick hairy 1 in mammal to carry out transcription regulation	Sheeba et al. (2007)
SAP25	Besides stabilizing Sin3 core complex, it is also involved in transcription repression mediated by Sin3A in mammals by interacting with different transcription factors	Shiio et al. (2006)
SAP30	Proteins of the SAP30 family (SAP30 proteins) have a functional nucleolar localization signal and they are able to target Sin3A to the nucleolus in mammals, also interact with transcription factor and co-repressor to bring transcription regulation. Interacts directly with Rb-associated polypeptides and with HDAC1and help in stabilization of core complex	Viiri et al. (2009)
SAP45/SDS3	Important for the integrity and catalytic activity of the Sin3/HDAC core complex	Grzenda et al. (2009)
SAP130	Besides stabilization of Sin3A core complex, it can also interact with mSin3A- and HDAC-independent co-repressors and transcription factors	Fleischer et al. (2003)
SAP180	Involved in stabilizing the mSin3A complex on DNA and in mediating interactions between mSin3A and HDAC-independent co-repressors	Fleischer et al. (2003)
RPB1	Repressor for the key tumor suppressor gene Rb; functions in senescence and development and cell cycle. Interacts directly with SAP30 thus helps in stabilizing SAP30 assembly in Sin3 complex	Xie et al. (2011)
$p33^{ING1b}$	Like RBP1, it also directly interacts with SAP30 and helps in stabilization SAP30 on Sin3 core complex. Interactions between p33ING1b and Sin3A are also required for the antiproliferative function of cell	Kuzmichev et al. (2002)
BRMS1	Suppresses metastasis of multiple human and murine cancer cells without inhibiting tumorigenicity. Directly bind to SAP30 of Sin3A core complex in mammals, helps in maintaining of the core complex	Meehan et al. (2004)

(continued)

Table 1.1 (continued)

Component of Sin3 complex	Functions	References
ING1/ING2	Directly binds to SAP30 of Sin3 core complex in mammals, helps in maintaining of the core complex. Together with p33ING1b function to repress cell proliferation and import of proteins in nucleus	Grzenda et al. (2009)
MeCP2	Methy-CpG-binding protein involved in the long-term repression of genes during mammalian development in Sin3A. Interact directly with HDAC1 of Sin3 core complex helps in stabilizing the interaction between HDAC1 and HID domain	Nan et al. (1998)

Fig. 1.1 The yeast Sin3 protein: Contains 1,536 amino acids and has six regions that are highly conserved throughout evolution. The PAH (paired amphipathic helix) domains appear to be protein-interaction domains, separated by spacer. The HID (Histone interacting domain) region between PAH3 and PAH4 interacts with HDACs and many of the other core components of the Sin3/ HDAC complex. The HCR (Highly conserved region) was recently identified as another protein-interaction domain that resides on C-terminal region of Sin3 protein

canonical PAH hydrophobic cleft while two other helices and an N-terminal extension target a discrete surface of the PAH3 domain formed by α helices (α2, α3 and α3′) (Xie et al. 2011).

In spite of substantial structural homology and similarity between PAH1 and PAH2 domains of Sin3 in various organisms, the two domains recognize different sequence motifs, thereby enabling differential target specificity. However, PAH3 domains share relatively low levels of sequence identity with PAH1 and PAH2 domains (25 and 16 %, respectively), yet these domains are structurally homologous to one another (Xie et al. 2011). Recent studies from our lab have shown that the three PAH domains, although showing homology in the tertiary structure, respond differentially to the environmental signals (pH and temperature). We found that PAH2 and PAH3 domains of Sin3B largely exist in unfolded state and are thermodynamically unstable in nuclear pH condition with respect to physiological pH while the structural identity of PAH1 remains unaltered at both the pH values (Tauheed Hasan et al. 2015). Based on these studies, we assume that there exists a flexibility and conformational heterogeneity in structure of Sin3 which provides additional new surfaces for protein-protein interactions.

1.4 Isoforms of Sin3

The basic protein structure of Sin3 is highly conserved among eukaryotes from yeast to mammals and has a varied number of isoforms (Fig. 1.2). *Saccharomyces cerevisiae* has only one isoform of Sin3 while *Schizosaccharomyces pombe* has three isoforms Pst1, Pst2 and Pst3, each encoded by a separate gene. Study on three paralogues of fission yeast (*Sch. Pombe*) revealed that these isoforms have originated due to gene duplication. Pst1 is most closely related to Sin3 of budding yeast (*S. cerevisiae*), suggesting that gene duplication occurred early in the evolution process. Pst2 appears to have arisen from Pst1 gene duplication during evolution of fission yeast. Pst3 seems to have arisen from duplication very early in evolution (Benedik et al. 1999). In Drosophila, three isoforms of Sin3 (Sin3 187, Sin3 190 and Sin3 220) are reported, which are produced by alternate splicing, having different C-terminals. Sin3 220 is expressed in proliferating cells

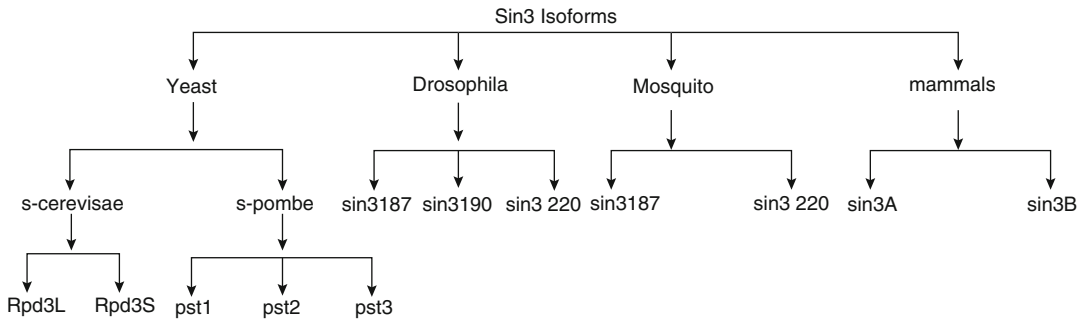

Fig. 1.2 Isoforms of Sin3 so far reported in eukaryotes

whereas Sin3 187 is expressed in differentiated tissues. The adult females and embryos contain Sin3 190. Thus, each isoform expresses in different cells and tissues and regulates different sets of genes and therefore can perform non-overlapping functions. Multiple Sin3 isoforms have also been reported in three species of mosquitoes which are conserved at splice sites and have a similar protein sequence to that of Drosophila Sin3 187 and Sin3 220 (Sharma et al. 2008).

In mammals, two isoforms of Sin3 are present, Sin3A and Sin3B, which are encoded by two separate genes (Ayer et al. 1995). These isoforms are results of gene duplication. The mouse Sin3A is more closely related to human Sin3A than to mouse Sin3B. Mammalian Sin3A and Sin3B proteins are approximately 57 % identical throughout the length of their polypeptide chains with the highest degree of homology localized in the PAH and HID suggesting that the duplication of Sin3 in mammals occurred before speciation (Alland et al. 1997; Silverstein and Ekwall 2005; Yang et al. 2000). The two isoforms also differ in the length of their polypeptide chain. Sin3B has a shorter amino acid tail prior to PAH1 with respect to Sin3A. Both Sin3A and Sin3B are widely expressed, frequently within the same cells and tissues, and interact with common as well as distinct transcriptional repressors and complexes. Splice variants of both the isoforms have also been reported in mice and humans. One example is mouse $Sin3B_{SF}$ (Sin3 B short form), in which the first 327 amino acids are identical to those present in long-form mSin3B ($Sin3B_{LF)}$ but are followed by a unique 19-residue stretch in the C-terminus, resulting in a truncated mSin3B with intact PAH1/2 domains but lacking the remainder of the C-terminus portion, including PAH3 and HID. It is envisaged that the splice variant forms perform differential function in same types of cells and tissues. $Sin3B_{SF}$ provides a more attenuated and reversible type of regulation on the basal transcriptional apparatus, while $mSin3B_{LF}$ acts as a nucleosomal condenser to provide more effective multi-level gene repression (Alland et al. 1997; Nair and Burley 2006). *Xenopus* Sin3 has one isoform which shows homology to Sin3A, revealing duplication of genes before divergence to vertebrates (Silverstein and Ekwall 2005). Isoform of Sin3 in *C. elegans* shows a unique structural hierarchy, having only one PAH domain in contrast to four PAH domains of Sin3 in other eukaryotes. This PAH domain shows 50 % sequence similarity with PAH1 domain of mammalian Sin3A. Thus, PAH domain of *C. Elegans* is structurally and functionally more evolved than Sin3 of higher organisms (Wysocka et al. 2003).

Purification and characterization of the components of the Sin3 complex by our group have shown that these isoforms contain different subsets of protein on their core complex. The different complexes show some common sub-unit whereas other sub-units are specific to each complex (Table 1.2). The mechanism of regulation of genes is also different in these complexes. For example, in yeast (*S. cerevisae*), two distinct complexes, large Rpd3L and small Rpd3S, are present. Although sub-units such as Sin3, Rpd3 and Ume1 are shared by both the complexes, Dep1, Pho23, Rxt1, Rxt2, Sds3 and Sap30 are specifically present in Rpd3L complex while

Table 1.2 Distinct subunits associated with Sin3 protein in different organisms (Yang and Seto 2008)

			S. cerevisae		*S. pombe*		
Mammals	*D. melanaogaster*	*C. elegans*	Rpd3L	Rpd3S	Complex I	Complex II	Protein domain
HDAC1, HDAC2	RPD3	HDA-1	RPD3	RPD3	Clr6	Clr6	ClassI deacetylase
RbAp46/RbAp48	p55	RBA-1, LIN 53	Ume1	Ume1	Prw1	Prw1	WD40 repeat
Sin3A or Sin3B	Sin3	Sin3	Sin3	Sin3	Pst1	Pst2	PAH motifs
Sds3, BMRS1	Sds3		Sds3		Sds3		Coiled coil
SAP30							
Sap18			Sap30				
ING1/2							Ubiquitin fold
			Pho23		Png2		PHD finger
				Rco1			PHD finger
				Eaf3			Chromodomain
						Cph1	PHD finger
						Cph2	PHD finger
						Alp13	Chromodomain

Rco1 and Eaf3 are unique sub-units of Rpd3S complex (Keogh et al. 2005). The mechanism of repression for each complex is also distinct; the Rpd3L complex represses gene expression by binding to the promoter through DNA-binding factors or other co-repressors. In contrast, the Rpd3S-mediated trans-repression is complex and involves recognition of methylated H3K36 by chromo-domain of Eaf3 and subsequent recruitment of core complex. This in turn brings about deacetylation of nucleosomes of the transcribed genes. Furthermore, during transcription elongation, Rpd3S complex enhances histone deacetylation thereby preventing transcription initiation from the intragenic cryptic promoters (Li et al. 2007). A similar repression mechanism has also been observed in *S. pombe*, wherein Pst1 and Pst2 form two distinct complexes (Complex I and Complex II). Complex I contains Pst1 and Sds3, and complex II encompasses Pst2, Alp13, Cph1 and Cph2 as distinct subunits whereas Clr6 and Prw1 are common to both the complexes (Nicolas et al. 2007). Likewise in Drosophila, Sin3 187 and Sin3 220 isoforms form two discrete complexes that are localized on distinct regions of polytene chromosome (Spain et al. 2010). Recently, a homologue of Rpd3S complex comprising of Sin3B, HDAC1, Mrg15 and Pf1 was reported in mammals. This complex mediates restoration of repressed chromatin structure at specific actively transcribing loci approximately 1 kb downstream of transcription start site (Jelinic et al. 2011). The above-mentioned examples of unique regulatory functions for each Sin3 isoform justify the concept of gene duplication to achieve complete functional plasticity during evolution.

1.5 Functional Aspect of Sin3A and Sin3B

Human Sin3A (hSin3A) is more homologous to mouse Sin3A (mSin3A), suggesting that divergence of Sin3A and Sin3B from Sin3 gene occurred before speciation during evolution (Halleck et al. 1995; Silverstein and Ekwall 2005). Interestingly, both Sin3A and Sin3B are expressed ubiquitously and are often present in same types of cells and tissues (Silverstein and Ekwall 2005). Since both Sin3A and Sin3B are evolutionary conserved, at least some of the functional aspects of each isoform must be specific and unique. Differential expression and interaction with distinct protein components would provide extensive flexibility both during embryonic development and normal cellular homeostasis. Studies have indicated that Sin3A is essential in early embryonic development of eukaryotes whereas Sin3B is essential for late embryonic development (Brunmeir et al. 2009; McDonel et al. 2009). It is also reported that Sin3A and Sin3B target similar as well as unique sets of genes. DNA-binding transcription factors like KLF, Mad1, REST and ESET are observed to interact with both isoforms. On the one hand, transcription regulators SMRT and MeCP2 associate with mSin3A while the master regulator of MHC II, CIITA mediates IFN-γ induced repression of collagen type I gene transcription via HDAC2/Sin3B (Kong et al. 2009; Nagy et al. 1997; Nan et al. 1998). Sin3B was associated with repression of subsets of E2F target genes while both Sin3A and Sin3B interact with p53 and regulate its target gene repression. Sin3B also plays an essential role in promoting the cell cycle via the E2F–Rb pathway (Bansal et al. 2011; Blais and Dynlacht 2007; Kadamb et al. 2013). In fact, Sin3B-deficient mice confirmed the role of mSin3B in cell cycle exit control and repression of E2F target genes *in vivo*, along with a role in the differentiation of erythrocytes and chondrocytes. Sin3B is upregulated during oncogenic stress signalled by Ras overexpression and that expression of Sin3B was required for cellular senescence (David et al. 2008). In contrast, removal of Sin3A did not affect cell cycle progression; it instead promoted apoptosis of breast cancer cells MCF7. These observations reinforce the idea that each isoform of Sin3 has several unique targets in the cell (Hurst 2013).

1.6 Sin3-Protein Interactions

Sin3 targets chromatin either through direct interaction with DNA-binding transcription factors or indirectly through another adaptor molecule, known as co-repressor complex. A large number of transcription factors interact with Sin3 through its six conserved domains that include the four PAH1-4, HID and HCR. NMR and crystallographic structural data available so far for Sin3 suggest that N-terminal region of Sin3, which includes PAH1-3 domains of Sin3, shows conformational heterogeneity whereas the structure of C-terminal region of Sin3 (HID, PAH4 and HCR) is highly stable. This may be one of the reasons due to which PAH1-3 regions of Sin3 can interact with a large number of transcription factors, whereas very few transcription factors interact with C-terminal region of Sin3. Other factors such as difference in sequence of PAH domains, recognition of different SID domains (Sin3 interacting domain) by PAH domains, etc. make each PAH domain a unique region, capable of interacting with diverse transcription factors and co-repressors to regulate transcription. Thus, Sin3 acts as a master scaffold to provide a platform for the assembly of numerous transcription factors and co-repressors. Besides this, multiple domains within Sin3 serve as excellent protein-binding interfaces which in turn make Sin3 to be suitable for multiple protein interactions.

1.6.1 Interaction with DNA-Binding Proteins

The PAH domains of Sin3 are specialized for interaction with DNA-binding domains. These PAH domains are structurally homologous to one another, yet they recognize different sets of transcription factors or DNA-binding proteins due to the fact that these PAH domains have different amino acid sequences which recognize different sequence motifs of transcription factors to bring out transcription repression. Figure 1.3 shows CLUSTAL-Omega-guided multiple sequence alignment of PAH1, PAH2 and PAH3 of Sin3.

Majority of proteins so far identified to interact with sin3 interact with its PAH2 domain. Although PAH2 domain is the closest relative of the PAH1 domain having 30 % sequence identity and 54 % sequence similarity, only a few interacting partners are known to interact with PAH1 in contrast to PAH2, suggesting dissimilar modes of engaging targets. However, PAH3 domains share relatively low levels of sequence identity with the PAH1 and PAH2 domains having 25 % sequence identity and 45 % sequence similarity with PAH1 and 16 % sequence identity and 48 % sequence similarity with PAH2 domains respectively, yet it recognizes larger sets of proteins than PAH1 (Le Guezennec et al. 2006; Moehren et al. 2004; Sahu et al. 2008). Previous studies have also shown that PAH1 amino acid residues Val7, Leu14 and Lys39 play an important role in the specificity between PAH1 and SID domains whereas PAH2 residues Phe7, Val14 and Gln39 are important in determining the specificity between PAH2 and SID domains (Le Guezennec et al. 2006). Similar observations are lacking for PAH3 domains. Mutation in these amino acid sequences may decrease in binding specificity of SID (Sin3 interacting domain) domains.

Moreover, each PAH domain of Sin3 recognizes different sequence motifs on DNA-binding proteins of Sin3 which is also known as SID domain (Sin3 interacting domain). Initial attempts to identify a SID consensus sequence for Mad family members in PAH2 revealed the following degenerate sequence: φφZZφφXAAXXφnXXn with X for any non-proline residues, φ for bulky hydrophobic residues and n for negatively charged residues. Other studies have reported the SID of SP1-like members with PAH2 as φφXAAXXφ. Similarly in case of PAH1, LXXLL consensus sequence was identified as SID of SAP25, a component of the Sin3 complex. Thus by performing a yeast two-hybrid screening of a peptide aptamer library, a small repertoire of peptides interacting specifically either with PAH1 or PAH2 domain of mSin3B was identified, which is listed in Table 1.3 (Le Guezennec et al. 2006).

```
PAH3      QEVYENFLRCIALFNQELVSG-----------SELLQLVSPFLGKFPELFAQFKSFLG
PAH1      PATYNGFLEIMKEFKSQSIDTPG-----------VIRRVSQLFHEHPDLIVGFNAFLP
PAH2      PEIYRSFLEILHTYQKEQLNTRGRPFRGMSEEEVFTEVANLFRGQEDLLSEFGQFLP
             *$ **$ #  ##$# #$             ##  *# ##    #*#  *  **
```

Fig. 1.3 CLUSTAL O (1.2.1) Multiple sequence alignment of PAH1, PAH2 and PAH3 of *Homo sapiens* in Sin3B. *represent identical amino acids whereas # and $ represents similar and non-polar amino acids respectively

Table 1.3 Peptide aptamer library binding to PAH1 and PAH2 domains of Sin3B (Le Guezennec et al. 2006)

Sequence binding to the PAH1 domain of mSin3B	Sequence binding to the PAH2 domain of mSin3B
1. EGDWLFFVLLVGLL[a]	1. NLGLLVLGSVCTFRLGLL[a]
2. LACRCWRCVHTRVRLLW[a]	2. VDSAWAALLSVACCSLWGLV
3. VDVYGSFGLLWCFECFVSEC	3. EGCVVVDWAAWVLVGYAAGW
4. LWLLSEVSSVYGT[a]	4. MPSSLNFGSFMGASLWGPLVIAFFLRFRRTGLFLLWVV[a]
5. SLALYASIWLLAEHWPQGLI	5. WWAWLSVKLGHSGDWLVSLF
6. WSLTWVPLYVLTMCAYWRSL	6. VVFLCFLDSLSCAVDAAGDD
7. DRLSCFWCWWLVFLTHHAAG	7. GVFFVFLLWTSMPSAESTSM
8. AAMGSAEFEALVALLFLCEE	8. SSPYCSSRWKILSF[a]
	9. FYLWMLSRGGSGPMLLQIVL
	10. CMWLMGHSMRLILIWCACMT
	11. VEHCDFCCTVAALVLMSRES

[a]Indicates a truncated peptide

Structural flexibility and conformational heterogeneity of PAH domains are also two of the major factors which help in recruiting different sets of DNA-binding proteins onto the PAH domains of Sin3. NMR and X-ray crystallographic structures of PAH1 and PAH2 have shown that these are structurally independent and fold in a different way to interact with the SID domains of the peptides (Guezennec 2006; Swanson et al. 2004). In the complex, PAH1 holds a left-handed four-helix bundle structure followed by a semi-ordered C-terminal tail; however, PAH2 domain adopts a wedge-shaped helical conformation in the complex engaging the cleft through a non-polar surface (Nomura et al. 2005). Essentially, the architecture of the four helices of PAH1 is similar to the corresponding structure of the PAH2 domains, except that they are shorter (Zhang et al. 2001). There also exists a structural heterogeneity in orientation of PAH domains on binding with different transcription factors. Mad1 and HBP1 trans-repression domain binds through a helical structure to the hydrophobic cleft of mSin3A PAH2, but the HBP1 helix binds PAH2 in a reversed orientation relative to Mad1 (Swanson et al. 2004).

Presence of different isoforms and splice variants of Sin3 in various organisms further add to the structural complexity and heterogeneity of PAH domains to interact with diverse binding partners. Though PAH2 domains of both mSin3A and Sin3B proteins interact with their targets in an analogous manner, the non-interactive forms of these two paralogues differ in their structure. The PAH2 domain of apo-mSin3A homodimerizes and exists in unfolded state, but apo-mSin3B PAH2 domain is monomeric and is fully folded (Kadamb et al. 2013).

Structures of PAH3 complex with SAP30 have been recently characterized, which suggests that PAH3 domain also forms a four-helix structure similar to PAH1 and PAH2 domains of Sin3 on interaction with SID domain of SAP30 (Xie et al. 2011). However, the individual structure of PAH3 domain is yet to be characterized by NMR and X-ray crystallography. Recent study from our lab suggests that PAH3 and PAH2 domains of Sin3B largely exist in unfolded state whereas PAH1 exists in folded state in nuclear pH conditions. The flexibility in the structure of PAH2 and PAH3 at nuclear pH condition allows them to interact with large numbers of binding partners

(Tauheed Hasan et al. 2015). Transcription factors have also been reported to interact with two different regions of Sin3 thus giving the next level of complexity in the interaction of proteins. Table 1.7 shows proteins interacting with two different regions of proteins.

Few transcription factors have also been shown to interact with regions of Sin3 not predicted to form amphipathic helices such as HCR, HID and PAH4. The PAH4 domain of Sin3 is highly conserved throughout in the length of their polypeptide in different organisms and exists in a folded state and thus does not show any structural heterogeneity (Cowley et al. 2004; Kadamb et al. 2013). Only one interacting partner has been known so far in case of PAH4 domain. Like PAH4 domain, HID and HCR regions of Sin3 are also highly conserved in different organisms. Thus, it is believed that N- terminal of Sin3, which includes PAH1-3, is involved in interacting with different DNA-binding transcription factors, whereas C-terminal part which includes HID, PAH4 and HCR is involved in the scaffolding function of Sin3 by serving as interaction site for other subunits of the co-repressor complex.

The first transcription factor characterized as interacting with Sin3 was Mad1. The Mad1 repressor belongs to a family of four proteins (Mad1, Mxi1, Mad3 and Mad4) that are thought to antagonize the transcriptional activation, proliferation-promoting and transformation functions of the oncoprotein Myc and thereby acts as tumour suppressor (Pang et al. 2003). Solution structures of the PAH2 domain complexed to the Sin3 interacting domain (SID) of Mad1 showed that the complex is folded as a 'wedged helical bundle', in which the PAH2 domain adopts a four-helix bundle conformation in which the α-helix of the SID is inserted (Brubaker et al. 2000). Till now, numerous binding partners have been discovered that interact with Sin3 for transcription regulation. A brief list of the proteins interacting with different domains of Sin3 are given in Tables 1.4, 1.5, 1.6, 1.7 and 1.8.

Earlier, Sin3 was thought to be a transcription repressor, but many transcription factors are able to positively regulate gene expression. The first report of Sin3 as an activator was published in the 1990s wherein it was found to activate GAM3, a gene encoding an extracellular glucoamylase in yeast, through interaction with STA1 (transcription factor) (Yoshimoto et al. 1992). Another interesting example is the heat stress-induced Sin3-mediated gene activation (CTT1, ALD3, PNS1 and TPS1) (Ruiz-Roig et al. 2010). Further studies are required to understand the mechanism of Sin3-mediated gene regulation.

1.6.2 Interaction of Proteins with Members of Sin3 Core Complex and Co-repressor Complex

Other targeting peptides interact with Sin3 in a more indirect manner to bring transcription regulation. They may interact through one or several members of the core complex, or indirectly via

Table 1.4 List of some of the proteins interacting with PAH1 domain of Sin3

Transcription factors	Function	References
PLZF	Restrict proliferation and differentiation of core blood derived myeloid progenitors to maintain a balance between progenitor and mature cell compartments in mammals	Guidez et al. (1998)
Opi1	Repressor of phospholipid biosynthesis in *S. cerevisiae*	Wagner et al. (2001)
Myt1	Regulate a critical transition point in oligodendrocyte lineage development by modulating oligodendrocyte progenitor proliferation relative to terminal differentiation and up-regulation of myelin gene transcription in mammals	(Romm et al. (2005)
HCF1	Repressor of cell proliferation in metazoans	Wysocka et al. (2003)

Table 1.5 List of important proteins interacting with PAH2 domain of Sin3

Factors	Function	References
Mnt	Novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites in mammals	Hurlin et al. (1997)
MDL-1	Homologue of vertebrate MAD protein involved in *C. elegans* cell proliferation and differentiation	Yuan et al. (1998)
Rox	Novel protein expressed in quiescent cells of mammals that heterodimerizes with Max, binds a non-canonical E box and acts as a transcriptional repressor	Meroni et al. (1997)
TGIF	A protein required for normal craniofacial development in mammals	Hui Ng and Bird (2000)
Ume6	Negatively regulates meiosis-specific genes in *S. cerevisiae*	Kadosh and Struhl (1997)
REST/NRSF	Repression of neuronal genes in non-neuronal tissues in yeast and mammals	Coulson (2005)
Sp1-like transcription factors	Family of repressor proteins which regulate mammalian cell homeostasis	Kaczynski et al. (2003)
HBP1	Transcriptional repression of a cell cycle inhibitor and regulator of differentiation in mammals. Present only in Sin3A	Swanson et al. (2004)
Foxk1	Important transcription factor in the myogenic progenitors in mammals	Shi and Garry (2012)
TIS7	Regulates epithelial cell polarity in mouse mammary glands in mammals. Present only in Sin3B	Vietor et al. (2002)

special adapter molecules called co-repressors. Sin3-associated proteins (SAPs) are important members of core complex that play a vital role in

Table 1.6 List of some of the proteins known to interact with PAH3 domain of Sin3

Factors	Function	References
AF1	Domains of the Androgen Receptor Interact with Distinct Regions of SRC1 and negatively regulates the transcriptional activities of hPR-A and hPR-B	Nawaz et al. (1994)
HAP1	RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription	Nawaz et al. (1994)
ETO	acute myeloid leukaemia, represses transcription	Hug and Lazar (2004)
AML1	Acute myeloid leukemia1 repress hematopoietic genes	Wang et al. (1999)
Elk-1	MAPK-inducible transcription factor involved in the up-regulation of immediate-early genes in response to growth factor and their subsequent repression	Silverstein and Ekwall (2005)

interaction with DNA-binding proteins. Binding with transcription factors results in conformational change and altered binding of SAP with PAH domains of Sin3 and consequent tethering of SAP onto the promoter of DNA. Since SAP also directly binds to HDAC, the SAP-Sin3-HDAC complexed with DNA results in histone deacetylation and thus repression of the genes (Zhang et al. 1998). The first member of SAP protein discovered to interact with transcription factors is SAP30, which specifically interacts with Sin3A isoform (Laherty et al. 1998). SAP 30 interacts with transcription factors such as papillomavirus binding factor (PBF), a nuclear-cytoplasmic shuttling factor which represses the cell growth. On interaction with PBF, SAP 30 is recruited on PAH3 domain of Sin3A to bring about transcription regulation (Sichtig et al. 2007). Solution structure of SAP30-PAH3 complex revealed that SID domain of SAP30 binds to PAH3 via a tripartite structural motif, including a C-terminal helix that targets the canonical PAH hydrophobic cleft while two other helices and an N-terminal extension target a discrete surface formed largely by the PAH3 α2, α3 and α3′ helices (Xie et al. 2011). SAP protein is also recruited

Table 1.7 List of the proteins interacting with more than one domain or regions of Sin3

Factors	Interaction surface on Sin3	Function	References
Ume6p	PAH2:PAH3	DNA binding protein that repress early meiotic gene (EMG) in *S. cerevisae*	Goldmark et al. (2000)
Ume6p	PAH3:PAH4	DNA binding protein that activate early meiotic gene (EMG) in *S. cerevisae*	Goldmark et al. (2000)
Pf1	PAH1:PAH2	Links Sin complex with groucho/TLE complex to globally repress gene expression in mammals.	Yochum and Ayer (2001)
MeCP2	PAH3:HID	Methyl-CpG-binding protein involved in the long-term repression of genes during mammalian development. Interacts only with Sin3A	Grzenda et al. (2009)
Ebp1	PAH4:HID	Inhibits the proliferation and induces the differentiation of human breast and prostate cancer cell lines in mammals. Interact selectively with Sin3A	Zhang et al. (2005)
P53	PAH2:PAH3	Tumor suppressor protein that governs apoptosis and cell cycle in mammals	Grzenda et al. (2009)

Table 1.8 List of the proteins interacting with the regions that do form amphipathic helixes

Factors	Interaction surface on Sin3	Function	References
OGT	PAH4	An O-GlcNAc transferase repressor in mammals	Grzenda et al. (2009)
DPE2(DNA polymerase epsilon)	HID	DNA polymerase epsilon is essential for cell viability and chromosomal DNA replication in budding yeast, also involved in DNA repair and cell-cycle in mammals	Grzenda et al. (2009)
Alien	HCR	Repressor for nuclear hormone receptors in mammals	Moehren et al. (2004)

directly by PAH domains onto the promoter of transcription regulation machinery to bring about transcription regulation. For example, SAP30 and SAP25 of core complex function as DNA-binding proteins that specifically bind to the promoter region of genes. Solution NMR structure of SAP25 and PAH1 domain shows that SAP25, highly unstructured, except for portions of the SID interacting segments, adopts a helical conformation. SAP25 binds through an amphipathic helix to a predominantly hydrophobic cleft on the surface of PAH1 (Sahu et al. 2008). Thus, SAP25 and SAP30 adopt a different fold to get recruited by PAH1 and PAH3 domains of Sin3 to bring about transcription regulation. Other members of the Sin3 complex recruited directly or indirectly by different PAH domains of Sin3 are listed in Table 1.9.

Co-repressors typically serve as link between the DNA-binding proteins and Sin3/HDAC complex. However, co-repressors may augment transcriptional silencing through their intrinsic repressor activities, or even by recruiting auxiliary functionality. Nevertheless, addition of co-repressor to Sin3/HDAC complex alters the properties of the complex, sometimes drastically. The best studied co-repressors are associated with nuclear receptors: nuclear receptor

Table 1.9 List of factors recruited by some of the members of the Sin3 complex with different PAH domains of Sin3

Factors	Interaction surface on Sin3	Function	References
GAGA	SAP18:PAH2	Contribute to the regulation of homeotic gene expression in drosophila	Espinas et al. (2000)
Chick hairy 1	SAP18:PAH2	Responsible for somatogenesis in mammals	Sheeba et al. (2007)
PBF	SAP30:PAH3	Nuclear-cytoplasmic shuttling factor with the ability to inhibit cell growth in mammals	Sichtig et al. (2007)
SAP30	PAH3	Proteins of the SAP30 family (SAP30 proteins) have a functional nucleolar localization signal and they are able to target Sin3A to the nucleolus in mammals	Xie et al. (2011)
SAP25	PAH1	Involved in transcription repression mediated by Sin3A in mammals	Shiio et al. (2006)
SMRTER	PAH1	Drosophila co-repressor for the nuclear hormone receptor	Shi and Garry (2012)
REST/NRSF	PAH1	Repression of neuronal genes in non-neuronal tissues in yeast and mammals	Nomura et al. (2005)
N-CoR	PAH1; PAH3-N-terminal HID, N-terminal SAP30	Co-repressor for nuclear hormone receptors	Laherty et al. (1998)

co-repressor (N-CoR) and neural restrictive silencer factor (NRSF)/repressor element 1 silencing transcription factor (REST). These co-repressors provide a link between the Sin3/HDAC complex and nuclear receptors, a family of transcription factors that regulate gene expression in a ligand-dependent manner. Nuclear receptors recruit HAT and HDAC complexes in a context-dependent manner and thus facilitate both gene activation and repression without dissociating from the DNA (Alland et al. 1997; Nomura et al. 2005; Torchia et al. 1998). Thus, by simply changing its interaction partners, a transcription factor is able to modulate the transcriptional activity of genes.

In mammals, the N-terminal repressor domain of NRSF/REST interacts with PAH1 domain of Sin3B and represses neuronal gene expression in non-neuronal tissues. The NMR structures of the complex suggest that PAH1 holds left-handed, four-helix bundles with a semi-ordered C-terminal tail associated with a hydrophobic short α-helix of NRSF/REST. The NRSF/REST short helix is sandwiched between α1 and α2 helices of PAH1 and positioned at an angle of about 55° relative to the α2 helix. While in case of nuclear hormone receptor co-repressor (N-CoR), the vital structure of the four helices of PAH1 is similar to that of corresponding structure of the PAH1-NRFS/REST domains, but the affinity of binding of N-CoR to the PAH1 domain is low. One of the reasons could be that C-terminal region of N-CoR contains fewer hydrophobic amino acid residues than the NRSF/REST helix (Nomura et al. 2005; Wolffe 1997). Thus, for strong binding to a repressor, PAH1 preferentially requires a short α-helix with a large number of hydrophobic amino acid residues within the repressor. The conformation flexibility in structure of PAH domains binds co-repressors with different binding affinities thereby providing additional flexibility for regulating the promoter activity. For some transcription factors, special adapters have been developed that can mediate or stabilize the contact between these proteins and Sin3/HDAC. For instance, the adapter molecule RBP1 interacts with the nuclear phosphoprotein Rb and also serves to recruit Sin3/HDAC by interacting with the core component SAP30 and Rb protein (Lai et al. 2001).

1.7 Altered Structure of Sin3 Protein

1.7.1 Alteration in Structure Due to Post-Translation Modification of Sin3 Protein

The Sin3/HDAC co-repressor complex can be recruited by a large number of DNA-binding transcription factors or co-repressors, thereby requiring a precise and coordinated mechanism to achieve specific and timely regulation of transcription. Although poorly investigated, post-translational modifications in Sin3 protein play a very important role in fine-tuning of its regulation. Sin3 protein contains several potential sites for post-translation modifications such as phosphorylation, myristoylation, ubiquitination and SUMOylation (Bansal et al. 2011).

1.7.1.1 Phosphorylation

Protein phosphorylation is a post-translational modification of proteins in which a serine, threonine or a tyrosine residue is phosphorylated by a protein kinase by the addition of a covalently bound phosphate group. Sin3 phosphorylation by tyrosine kinase at key tyrosine residue helps to regulate protein stability through PAH domain-mediated protein-protein interactions (Bansal et al. 2011). For example, under normal conditions, levels of p53 (tumor suppressor protein) are tightly regulated by MDM2-mediated degradation. However, in case of cellular stress such as DNA damage, the levels of p53 increase significantly due to its stable interaction with phosphorylated sin3A. Interaction of p53 with Sin3A effectively masks the MDM2-binding motif and protects p53 from proteasome-mediated degradation in a manner independent of MDM2 (Bansal et al. 2011; Honda et al. 1997). Thus, under conditions of cellular and/or genotoxic stress, phosphorylation of Sin3A can mediate stabilization of its interacting partner proteins.

1.7.1.2 Myristoylation

Myristoylation is an irreversible protein lipidation modification where a myristoyl group, derived from myristic acid, is covalently attached by an amide bond to the alpha-amino group of an N-terminal glycine residue. Myristic acid is a 14-carbon saturated fatty acid (14:0) with the systematic name of *n*-Tetradecanoic acid (Farazi et al. 2001). In *Saccharomyces cerevisiae*, myristoylation status of Sin3 is used for regulation of Opi1 transcription factor which regulates biosynthesis of phospholipids. During glucose starvation, Sin3 gets myristoylated and recruits Opi1 transcription factors, which results in activation of Ino2p and Ino4p genes. Ino2p and Ino4p are basic helix–loop–helix (bHLH) transcription factors that form heterodimeric complexes which bind to inositol-choline response elements (ICRE, also known as UASINO). In presence of inositol, binding of Ino2p and Ino4p represses the biosynthesis of phospholipid (Chen et al. 2007). Thus, *Saccharomyces cerevisiae* can overcome glucose starvation by minimizing energy utilization by shutting down phospholipid biosynthesis.

1.7.1.3 Ubiquitination

Ubiquitination, a post-translational modification where ubiquitin (a small regulatory protein that has been found in almost all tissues of eukaryotic organisms) is attached to a substrate protein. The addition of ubiquitin can affect proteins in many ways: it can signal for their degradation via the proteasome, alter their cellular location, affect their activity and promote or prevent protein interactions (Peng et al. 2003). Ubiquitination is carried out in three main steps: activation, conjugation and ligation, performed by ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s), respectively (Kadamb et al. 2013). The result of this sequential cascade binds ubiquitin to lysine residues on the protein substrate via an isopeptide bond or to the amino group of the N-terminus via a peptide bond.

Sin3 protein gets attached to a novel ubiquitin ligase, RING finger protein 220 (RNF220), and gets transported to cytoplasm where proteosomal degradation takes place. Although the mechanism is not clear, it is postulated that RNF220 acts as an E3 ubiquitin ligase for Sin3B and can promote its ubiquitination (Kong et al. 2010). Sin3 protein is thought to be a nuclear protein

where it brings about transcriptional regulation of various genes. Thus, not only can ubiquitination of Sin3 protein alter gene regulation, but its movement to cytoplasm may provide it with novel functions inside the cell.

1.7.1.4 SUMOylation

SUMOylation is a post-translational modification involved in various cellular processes, such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress and progression through the cell cycle. SUMOylation is directed by an enzymatic cascade analogous to that involved in ubiquitination. In contrast to ubiquitin, SUMO (*S*mall *Ubiquitin-like Mo*difier) is not used to tag proteins for degradation. Mature SUMO is produced when the last four amino acids of the C-terminus have been cleaved off to allow formation of an isopeptide bond between the C-terminal glycine residue of SUMO and an acceptor lysine on the target protein (Pungaliya et al. 2007).

Recent studies indicated that SUMOylation of Sin3 helps in stabilization of interaction between retinoblastoma-binding protein 1 (RBP1) and SAP 30 in Sin3/HDAC complex. RBP1 is a cellular protein that interacts with the pocket of retinoblastoma protein (pRB) and appears to be an important factor in the repression of E2F-dependent transcription by RB family of proteins. pRB appears to function principally as a transcriptional repressor that blocks E2F-dependent transcription, thus regulating entry into the S phase of the cell cycle (Binda et al. 2006). This cellular proliferation pathway is deregulated in a vast majority of cancers. Thus, Sin3 SUMOylation may play an important role in suppression of cell proliferation.

1.7.2 Modification of Sin3 Structure Due to Protein-Protein Interaction

The Sin3 has a dual role, functioning not only as a molecular scaffold for complex assembly but also as a molecular adapter, bridging HDACs with an astonishingly large and diverse group of DNA-binding transcription factors and chromatin-binding proteins. Most of the protein-protein interactions are achieved through six conserved domains of Sin3 that include four paired amphipathic helices (PAH 1–4), one HID and one highly conserved region (HCR). However, most of them interact with PAH domains of Sin3, especially with PAH2 and PAH3. Although there is a high degree of similarity between the PAH domains, yet PAH domains recognize different sequence motifs thereby exhibiting high degrees of specificity for their target. The PAH domains mediate specific protein–protein interactions, most likely through their independent associations with various repressors. Upon interaction with the transcription factors, these PAH domains adopt diverse conformational folds that help PAH domain to recruit different binding partners. Of the four Sin3 PAH domains, the tertiary structures of only PAH1 and PAH2 domains have been determined by NMR.

The second copy of the PAH domain (PAH2) is the site for interactions with numerous factors of which some are listed in Table 1.5. Structural studies have revealed that the PAH2 domain of mammalian Sin3 exhibits conformational heterogeneity that enables it to interact with diverse protein targets. PAH2 domain of mSin3B protein interacts with its targets in a manner similar to that of mSin3A, but these two paralogues differ in their non-interactive forms. The apo-mSin3A PAH2 domain homodimerizes and exists in unfolded state, but apo-mSin3B PAH2 domain is monomeric and is fully folded (Kadamb et al. 2013). Solution structures of the PAH2 domain complexed to the Sin3 interacting domain (SID) of Mad1 have shown that the complex is folded as a 'wedged helical bundle', in which the PAH2 domain adopts a four-helix bundle conformation into which the α- helix of the SID is inserted (van Ingen et al. 2006). However, the HMG box-containing repressor HBP1, which targets several cell cycle-specific and differentiation-specific genes, was shown to interact with the PAH2 domain. The structure of this complex showed a reversed orientation of the SID relative to the Mad1 complex, while maintaining the overall fold. The reversal of this helix orientation is

correlated with a reversal in the SID sequence motif (Swanson et al. 2004). Furthermore, molecular dynamic simulation has suggested that TIEG2 SID binds PAH2 in a different orientation than the Mad SID, suggesting multiple ways to interact with PAH2 domain.

By contrast, less is known about the mode of interaction and recruitment of the Sin3 proteins via the PAH1 domain. The solution structures of the apo-mSin3A PAH1 domain adopts the canonical left-handed four-helix bundle fold while the SAP25 SID in the mSin3A PAH1 complex is largely unstructured, except for some segments that adopt a helical conformation (Sahu et al. 2008). However, PAH1 holds a rather globular four-helix bundle structure with a semi-ordered C-terminal tail on interaction associated with the NRSF/REST repressor domain (Nomura et al. 2005). Although the PAH2 domain is the closest relative of the PAH1 domain, the domains exhibit distinct patterns of sequence conservation, suggesting dissimilar modes of engaging targets.

However, the folded structure of PAH3 and PAH4 domains of Sin3 has not been solved yet. More recently, some of the interacting partners of PAH3 domain have been identified (see Table 1.6), suggesting that PAH3 domain of Sin3 also exhibits conformational plasticity upon interacting with proteins. Till now, only one interacting partner of PAH4 has been identified (Table 1.8), suggesting that this domain may not exhibit conformational flexibility. Thus, structural and dynamic plasticity in Sin3 PAH domains, upon protein –protein interaction, helps Sin3 to interact with large numbers of transcription factors.

1.8 Structural Allostery

Structural allostery refers to the biological phenomenon where ligand/effector binding or energetic perturbation at one molecular site results in structure or activity changes at a second distinct site. The site where the effector binds is termed as the allosteric site and is different from the protein's active site. Allosteric sites allow effectors to bind to the protein, often resulting in a conformational change. Effectors that enhance the protein's activity are referred to as allosteric activators, whereas those that decrease the activity of proteins are called allosteric inhibitors (Hilser et al. 2012).

Allostery in the structure of Sin3 protein helps to recruit unique sets of proteins onto different sub-domains of Sin3 to bring transcription regulation. As discussed earlier in this chapter, Sin3 acts as a scaffold, which provides platforms to various proteins and complexes to coordinate the transcription regulation of diverse genes. Structural allostery in Sin3 shows two types of modulation as under.

1.8.1 Allosteric Modulation

Allosteric modulation of a receptor results from the binding of allosteric modulators at a different site (a 'regulatory site') from that of the endogenous ligand (an 'active site') and enhances or inhibits the effects of the endogenous ligand. Under normal circumstances, it acts by causing a conformational change in a receptor molecule, which results in a change in the binding affinity of the ligand (Fig. 1.4) (del Sol et al. 2009; Hilser et al. 2012).

1.8.1.1 Positive Modulation

Positive allosteric modulation (also known as allosteric activation) occurs when the binding of one ligand enhances the attraction between substrate molecules and other binding sites (Fig. 1.4a) (Hilser et al. 2012). HDAC1 (histone deacetylase complex 1) and HDAC2 (histone deacetylase complex 2) bind to the highly conserved HID region of Sin3, bring allosteric change in the structure of PAH2 domain of Sin3B and form a ternary complex. The ternary complex interacts with Mad/Myc proteins (allosteric activation) that is able to direct trichostatin A (TSA, a HDAC inhibitor) sensitive repression to luciferase under the control of a Myc-binding element (Nan et al. 1998).

1.8.1.2 Negative Modulation

Negative allosteric modulation (also known as allosteric inhibition) occurs when the binding of

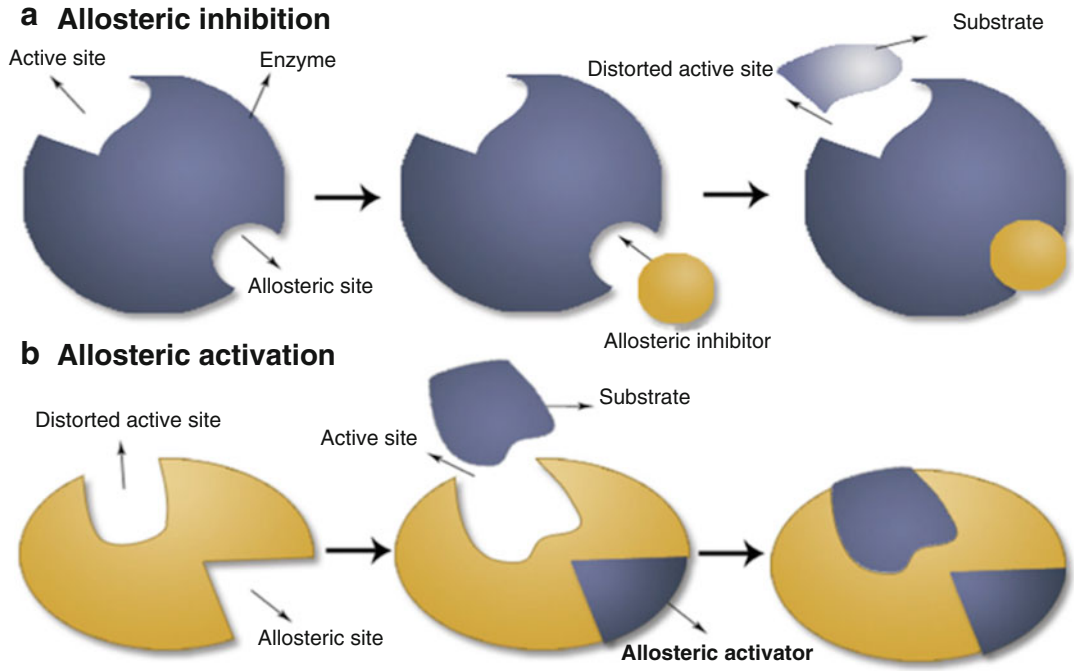

Fig. 1.4 Models showing Allosteric regulation of proteins

one ligand decreases the affinity for substrate at other active sites (Fig. 1.4b) (Hilser et al. 2012). Sin3/HDAC complex undergoes an allosteric conformational shift upon binding with reduced nicotinamide adenine dinucleotide (NADH) which in turn regulates the interaction of CtBP (C-terminal-binding protein) with REST co-repressor complex. High NADH levels cause CtBP to interact weakly with REST (negative modulation or allosteric inhibition, resulting in de-repression of transcription. However, low NADH levels allow CtBP to bind strongly with REST co-repressor complex and shut down transcription. Since the bulk of nuclear NADH is thought to be in equilibrium with cytoplasmic NADH and is derived in large part through glycolysis, lower rates of glycolysis result in reduced NADH, increased CtBP binding to REST and more repression of REST target genes (Chinnadurai 2002; Ooi and Wood 2007). This mechanism was exploited to control pro-epileptic gene expression in the kindling model using glycolytic inhibitors.

Structural allostery in Sin3 also helps in governing protein-protein interaction stability by which they can carry gene regulation. Phosphorylation of Sin3 by tyrosine kinase at key tyrosine residues induces conformational change in the structure of PAH2 domain. Upon phosphorylation, PAH2 domain forms α-helical pocket which interacts with SID domain of p53 (tumor suppressor protein). This interaction between SID domain of p53 and Sin3 complex alleviates proteasome-mediated degradation in a manner independent of MDM2 (Zilfou et al. 2001).

Similarly, NRSF/REST is a Kruppel-type zinc finger protein that mediates transcriptional repression through the association of its C-terminal repressor domain (RD-2) with CoREST/HDAC complex and N-terminal repressor domain (RD-1) with Sin3/HDAC complex. The N-terminal domain of NRSF/REST is folded into a unique hydrophobic α-helix which on interaction with Sin3 induces allosteric modification in unstructured C-terminal region of PAH1 domain such

that the latter adopts a left-handed four-helix bundle structure. This in turn stabilizes the binding of PAH1 to RD-2 domain of NRSF/REST. Both RD-1 and RD-2 interact directly with TATA-binding protein (TBP) via a chromatin-independent mechanism (Nomura et al. 2005). Several neurological diseases such as Down syndrome, medullo-blastoma and Huntington's disease are related with dysregulation of NRSF/REST and its target genes (Song et al. 2014).

1.9 Conclusion

Sin3 plays dual roles as a scaffold protein as well as adapter protein both in higher and lower eukaryotes. Due to the conformational diversity and structural flexibility of its different sub-domains, it is able to recruit a large number of DNA-binding proteins and co-repressor complexes. Various processes such as allosteric regulation, protein-protein interactions and post-translation modifications contribute towards allosteric structure and conformational diversity of Sin3. The flexible nature of Sin3 structure helps to interact with various proteins and facilitates regulation of diverse cellular processes inside the cell such as growth, differentiation and senescence as well as oncogenic transformation in pathological conditions.

1.10 Future Prospective

In spite of active research on the structure of Sin3 protein, the NMR structure of only PAH1 and PAH2 domains have been solved. Sin3 is able to recruit a large number of transcription factors and co-repressors due to flexible natures of different sub-domains. It will be imperative to decipher the function of different motifs present in the C-terminal regions of Sin3 so as to characterize and understand its interacting partners and their role in Sin3-mediated gene regulation. Future studies should also be directed towards understanding the regulation of Sin3 protein under different physiological conditions and modulation of its biological activity.

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Protein Post-translational Modifications: Role in Protein Structure, Function and Stability

2

Shilpi Mittal and Daman Saluja

Contents

Abstract

Protein synthesis and its folding to form quaternary structures are generally not sufficient to produce a functional protein. Proteins, soon after their translation, are often modified to achieve proper folding and localization. Some of the post-translational modifications include proteolytic cleavage, addition of prosthetic groups and addition of functional groups like phosphoryl, acetyl or methyl groups, the latter being reversible, and constitute an important role in modulating the activity of the protein. Post-translational modifications of proteins also regulate their stability as well as interactions with other proteins and macromolecules. Identification and understanding of the functions performed by the modified proteins is critical in the study of cellular homeostasis. It may provide new drug targets and also offer candidates for biomarker selection involved in chronic diseases. This chapter describes various post-translational modifications that occur in proteins and some of the key biological functions and signalling pathways controlled by these modifications in a living cell.

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Posttranslational modifications • Covalent modifications • Protein structure • Signal transduction • Proteolytic cleavage

2.1 Introduction

De novo synthesized polypeptide chains undergo various post-translational modifications (PTMs) that determine tertiary and quaternary structure of a functional protein. Post-translational modifications are divided into two groups. The first group involves cleavage of peptide bond, either by proteases or by autocatalytic cleavage that removes small polypeptide chains. The second group modifies side chains of amino acid residues by adding small chemical groups. Modifications generally include phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation and lipidation of certain aminoacids of a protein. Protein post-translational modifications increase functional diversity of proteins by adding these functional groups to the proteins. Enzymes involved in post-translational modifications in mammals include 500 protein kinases, 150 phosphatases and 500 proteases (Walsh et al. 2005). This constitutes 5 % of genomes of higher eukaryotes depicting that large percentage of proteomes is involved in various post-translational modifications, thus reinforcing the important role played by these modifications in maintaining cellular homeostasis.

There are four main groups of functions that require post-translational modifications – (1) some apoproteins require covalent binding of organic molecules/prosthetic groups to become active, (2) PTMs regulate the structural conformation of certain proteins and stabilize them, (3) tagging of proteins and mediating intracellular localization. Glycosylated proteins that are destined to be part of cell membranes or secretory in nature have carbohydrate chains attached to amide nitrogen of asparagine, threonine, serine or hydroxyl-lysine. N-glycosylation of protein occurs in endoplasmic reticulum and O-glycosylation in golgi bodies. (4) PTMs also regulate the biochemical processes of a protein (Knorre et al. 2009). Covalent modifications of some of the amino acids can change the chemical nature of a protein. For example, phosphorylation of serine or tyrosine residues can add negative charge to the protein. Phosphorylation and/or acetylation of DNA-binding proteins play a critical role in gene regulation. Lysine carries positive charge under physiological conditions, but its positive charge is masked by monomethyl groups. Though methylation doesn't remove the charge, it increases the bulkiness which reduces its hydrophilicity (Grant 2001). Several post-translational modifications are reversible in nature and differentially regulate the gene expression and cellular metabolism in response to various signals. Hence, analysis of proteins and their PTMs could provide an invaluable insight in the etiology of various diseases. More recently, several protein kinases are being looked into as potential drug targets. As a large number of post-translational modifications are known in biological systems, a comprehensive review of all PTMs is difficult. This chapter provides an overview on the most common types of PTMs using some of the well-studied proteins as examples.

2.2 Covalent Modifications

2.2.1 Phosphorylation

In eukaryotes, phosphorylation occurs on the hydroxyl groups of serine, threonine and less frequently on tyrosine residues of a protein (Fig. 2.1). However, in bacteria and fungi, phosphoHis and phosphoAsp are also present that are produced through two component signal cascades (Walsh et al. 2005). Addition of phosphoryl groups adds two negative charges to the protein at physiological pH and provides its capacity to form extensive hydrogen bonds with its four phosphoryl oxygens (Johnson and Lewis 2001). Phosphorylation is catalyzed by a group of proteins called kinases, and this family consists of large numbers of proteins constituting a kinome family (Manning et al. 2002). Protein kinase catalyzes transfer of phosphate groups from ATP to protein (Fig. 2.2). Kinase has two domains: amino terminal of polypeptide, rich in

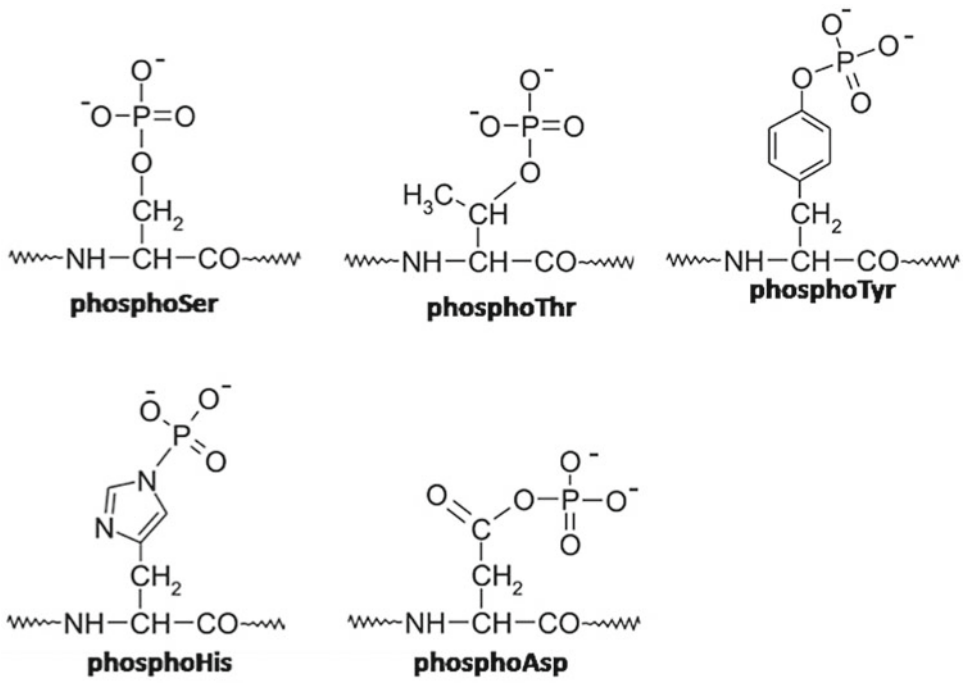

Fig. 2.1 Various amino acid residues that are phosphorylated in eukaryotes and prokaryotes

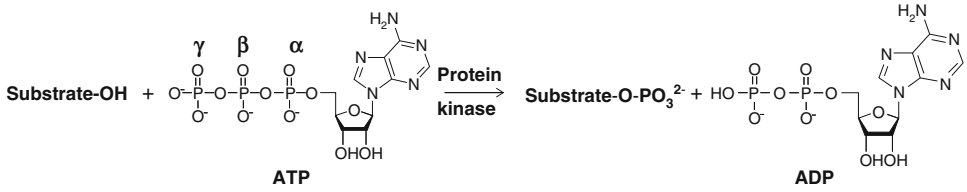

Fig. 2.2 Phosphorylation involves transfer of ϒ-phosphate from ATP to substrate in presence of protein kinase

β-sheet, binds ATP, while carboxy terminal, comprised of α-helix, is bound to protein substrate. However, dephosphorylation of phosphorylated proteins is catalyzed by phosphatase enzyme.

Phosphorylation in proteins generally occurs at multiple sites of proteins in response to various physiological signals (Xu 2003; Lindner 2008; Sarg et al. 2009). RNA polymerase II is an example of one such enzyme that gets phosphorylated at multiple serine and threonine residues of repeated heptapeptide consensus sequences (Tyr-Ser-Pro-Thr-Ser-Pro-Ser), which enhances its affinity to bind to various transcription factors.

Human p53 is another protein which gets extensively phosphorylated with 23 phosphorylation and dephosphorylation sites. Most of the phosphorylation is confined to serine and threonine amino acids that are concentrated at N-terminal trans-activation domain and C-terminal regulatory domain of p53. Importantly, the phosphorylation of most of these sites is specific to different stress conditions. p53 phosphorylation at S15/S20 residues of p53 not only stabilizes the protein and reduces its affinity for negative regulator HDM2 but also enhances its binding to co-activators (Gu and Zhu 2012). Phosphorylation of S46 in p53 is crucial for activation of pro-apoptotic genes whereas S392 phosphorylation initiates and stabilizes tetramer formation in response to UV induction (Matsumoto et al. 2006). It is interesting to note that p53 is not only phosphorylated under stress conditions but some phosphorylated residues are dephosphorylated in response to stress to mediate

p53 activation; serine 376 is phosphorylated in unstressed cells and dephosphorylated after ionizing irradiation promoting interaction of p53 with14-3-3 proteins. Phosphorylation is mediated at different residues by different protein kinases; the most important ones are ATM and ATR protein kinases. It has also been observed that a single site could be phosphorylated by more than one kinase and one kinase could phosphorylate more than one site (Kruse and Gu 2009). Nevertheless, these observations corroborate the fact that post-translational modifications regulate the functional activity of a protein (Toledo and Wahl 2006).

2.2.2 Acetylation

Acetylation of a protein occurs at the ϵ-amino group of lysine residues wherein acetyl coenzyme A acts as a donor of acetyl group and reaction is catalyzed by acetyl transferases. Addition of acetyl groups removes the positive charge from lysine residue, which changes the overall charge distribution of a protein and adds to the hydrophobicity of protein. Acetyltransferases are generally mentioned as histone acetyltransferases (HATs) as these are best known to acetylate histones, but protein substrates other than histones are also acetylated by HATs. The first HAT was isolated by Allis and co-workers in 1996 in *Tetrahymena* and was named as Gcn5 (Brownell et al. 1996). A plethora of HATs were then discovered within a decade, some of which had sequence conservation with Gcn5 while others had limited conservation (Marmorstein and Zhou 2014). Acetylation is a reversible process which is catalyzed by deacetylases called histone deacetylases (HDACs). There are 18 potential HDACs in mammals, classified as HDAC1 to HDAC11 and SIRT1 to SIRT7. Acetylation of N-terminus of histones and C-terminal of p53 is widely recognized. Histones are acetylated by wide groups of histone acetyltransferases that are divided into further subgroups. The octamer core of nucleosomes is comprised of two copies of each of histones H2A, H2B, H3 and H4, with 30 conserved lysine residues for acetylation (Khorasanizadeh 2004). Bromodomains, distinct domains present within transcription factors and associated proteins, recognize N-acetyl groups present on histones and thus initiate gene transcription (Walsh et al. 2005).

p53, a widely known transcription factor and tumor suppressor protein, is a major non-histone protein that is acetylated at nine sites, catalyzed by various HATs like p300, CREB-binding protein (CBP), p300/CBP-associated factor (PCAF), MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60 components), tat-interactive protein of 60 kDa (TIP60, also known as KAT5) and human Males absent On the First (hMOF). Six lysine residues (K370, K372, K373, K381, K382 and K386) in the C-terminal regulatory domain are acetylated by CBP/p300 that enhances the interaction of p53 with DNA. However, K320 acetylation is controlled by another acetyltransferase, PCAF, and promotes activation of genes involved in cell cycle arrest like p21 protein. Acetylation at K120 is induced upon DNA damage, and this modified p53 is recruited onto the promoters of pro-apoptotic genes but not recruited on the genes involved in cell cycle arrest. This suggests that K120 acetylation is important for mediating apoptosis in cells but plays no role in cellular arrest. K120 acetylated protein is also detected in mitochondria where it functions in regulating apoptosis by mediating BAK/MCL1 interaction (Sykes et al. 2009). Acetylation of p53 mediates its activation property by excluding ubiquitation on those sites, inhibiting recruitment of repressor HDM2/HMX complex on the promoters of target genes and recruiting co-activators. Acetylation at K120 and K164 appears to play a major role in controlling physiological functions as these sites are mainly mutated in cancer (Dai and Gu 2010). However, p53 is deacetylated by histone deacetylases (HDACs) like HDAC1 and sirtuin1 (SIRT1). SIRT1 preferably deacetylates K382 that inhibits p53 from activating apoptotic genes and mediating cell survival. p53 deacetylation by SIRT1 also inhibits its ability to activate cell cycle-arresting gene p21, which mediates normal cell cycling after DNA damage repair (Glozak et al. 2005).

It is quite interesting to note that these modifications are multi-potent in nature, i.e. a single modification can exert different responses under

different conditions. For example, K373/382 acetylation of p53 has been shown to activate p21 gene expression under certain conditions whereas others suggest that K373 acetylation has preference for regulation of pro-apoptotic genes (Zhao et al. 2006; Knights et al. 2006). It has been hypothesized that acetylation at one site may influence functioning and modification at other points.

2.2.3 Methylation

Methylation is another major post-translational modification that controls biological function of a protein and involves addition of one, two or three methyl groups to amino acid residues through methyltransferases. Amino acid residues that are most commonly methylated are lysine and arginine, but other aminoacids like histidine, glutamate, glutamine, asparagine and cystidine are methylated in some proteins. Methyltransferases are different from other PTM enzymes as these are specific not only to amino acid residues but also tertiary structure of protein substrates (Paik et al. 2007). Methylation of protein does not alter its charge but increases bulkiness and hydrophobicity of a protein that changes its inter- and intra-hydrogen bonding with interacting partners. Most of these enzymes constitute seven β-strand SET domains that recognize histones and non-histone proteins. The first methyltransferase identified possessed SET domain that mediates trimethylation of lysine 14 of enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) that is essential for fixing of CO_2 in plants (Klein and Houtz 1995). In mammals, a protein spanning seven β-strands, protein arginine methyltransferase1 (PRMT1), was discovered and found to methylate arginine specifically at Gly-Arg-Gly or Gly-Ala-Arg primary sequence. Subsequently, several other isoforms were identified (PRMT 1–9) depending upon primary sequence and tertiary structure of the substrates recognized by them. Studies regarding lysine methylation are mainly focused on histone methylation that controls gene expression. Methylation of certain lysine residues of histones like Lys36 and Lys39 causes euchromatin and gene activation whereas methylation of lysine residues like H3 Lys4, H3 Lys9, H3 Lys27 and H4 Lys20 mediates gene repression.

Non-histone substrates that are methylated by lysine methyltransferases involve transcription factors, heat shock proteins and receptor tyrosine kinases (Clarke 2013). Tumor suppressor protein p53 is also methylated at lysine and arginine residues. Arginine is methylated by PRMT5 at 333, 335 and 337 residues. Lysine in p53 can be monomethylated or dimethylated and can mediate gene activation or repression depending upon the site of modification. Moreover, functions of the modifications of the same sites vary according to the number of methyl moieties added (Carr et al. 2012).

2.2.4 Other Modifications

Glycosylation may modify OH groups of serine and threonine causing O-linked glycosylation or may modify side chains of asparagine leading to N-linked glycosylation. The oligosaccaryltransferase enzyme transfers a tetradecasaccharide (Glc3Man9(GlcNAc)2) fragment onto the target protein from the carbohydrate donor molecule dolichol pyrophosphate during N-glycosylation. Glycoprotein O-glycoside chains are much shorter and simpler than N-glycoside chains. O-glycoside chains of several proteins recognize transduction signals through their interaction with receptors (Knorre et al. 2009).

Another post-translational modification includes sulfation that adds sulfate to the -OH group of tyrosine. Phosphoadenosylphosphosulfate acts as a sulfate donor, and the reaction is catalyzed by the sulfotransferase enzyme. Sulfation of some receptor proteins increases their affinity for ligands (Knorre et al. 2009). Collagen protein undergoes hydroxylation, another post-translational modification, that adds OH groups to non-nucleophilic sites in amino acyl side chains to generate 3-OH-Pro, 4-OH-Pro and 5-OH-Lys and helps in protein

maturation. This modification is generally prevalent in secretory proteins and oxygen-sensing machinery. Addition of hydroxyl groups is catalyzed by prolyl hydroxylase though the enzyme required for removal of hydroxyl groups is not yet reported (Deribe et al. 2010).

2.3 Allosteric Regulation of Proteins by PTMs

PTMs in a protein regulate its tertiary and quaternary structure and thus control its functions. Additionally, PTMs alter the microenvironment of a protein and thus may change its interaction with other proteins. Phosphorylation of a protein inserts a dianionic, tetrahedral phosphate group in a protein that changes the conformation and enhances its affinity to bind to cationic arginine. These reorganizations caused by altered domains mediate signal initiations in the cell. Similarly, acetylation of carboxy terminal domain (CTD) of p53 mediates allosteric activation and exposes its DNA-binding domain resulting in recruitment of p53 onto the promoters of target genes, and its interaction is highly influenced by its conformation (Gu and Zhu 2012). Conformational changes through PTMs expose the buried docking sites or catalytic sites and thus induce the interaction between proteins, DNA and chromatin. Some of the proteins that are well studied are described below:

2.3.1 Structure Control by Phosphorylation

Addition of phosphoryl groups to serine, tyrosine or threonine can confer structural changes in a protein. The dianionic phosphoryl group has the capacity to form extensive hydrogen bonds. A phosphoryl group converts a hydrophobic residue to a hydrophilic one causing conformational changes in a protein by interacting with other hydrophilic and hydrophobic residues. Phosphorylation of a protein creates docking site in it that facilitates its interaction with other proteins.

2.3.1.1 CDK2 Phosphorylation

pCDK2 interacts with cyclinA to enter S phase of a cell cycle where it phosphorylates retinoblastoma protein (Rb) and other related proteins that regulate transcription. Studies on crystal structure of CDK2 revealed that ATP-binding site of protein is blocked. Addition of phosphoryl group to Thr160 of CDK2 causes structural changes in the protein that makes glycine-rich domain and activation domain more disordered. Thr160 resides near glutamate in an inactive CDK2, and insertion of a phosphoryl group mediates electrostatic repulsion causing disordered structure which is one of the important steps in its activity (Johnson and Lewis 2001).

2.3.1.2 Insulin Phosphorylation

Insulin binds to extracellular regions of insulin receptor that gets autophosphorylated at three residues, which then further phosphorylates exogenous substrates. Unphosphorylated insulin receptor kinase (IRK) possesses two lobes that are open by steric interactions, and the access to protein substrate and ATP is blocked. Upon phosphorylation, Thr1158 shifts to upper position of protein making no contact with protein and providing access for phospho-recognition sites to other proteins. pTyr1162 is displaced from the catalytic site, and hydrogen bonds to Arg1164. Tyr1163 is the last to be phosphorylated (Johnsonand Lewis 2001). The phosphorylation of these amino acid residues causes conformational changes in the insulin receptor protein that enhance interaction of substrate protein to its catalytic site.

2.3.1.3 STAT Proteins

STAT proteins comprise of signalling proteins that upon binding of cytokines mediate intracellular tyrosine phosphorylation. Phosphorylation causes STAT proteins to dimerize and cause their nuclear localization where they bind to promoters of its target genes. STAT has four domains: a four-helix coiled-coil domain, the DNA-binding domain, SH2 domain and C-terminus containing phospho-tyrosine. Structural studies of this protein reveal conformational changes upon phosphorylation that mediate protein dimerization and enhanced DNA binding (Becker et al. 1998).

2.3.2 Structural Regulation by Acetylation of Proteins

Acetylation of histones at lysine residues is associated with gene activation. Addition of acetyl groups to lysine residues removes positive charge and increases hydrophobicity of proteins that stabilizes β-hairpin-like conformation which reduces its interaction with DNA (Petty and Pillus 2013). Tetrahymena Gcn5 (tGcn5), a histone acetyltransferase, has higher affinity towards histone H3 than histone H4 and p53 substrates. The 15 lysine residues of histone H3 are ordered in structure as compared to that of histone H4 or p53 which have less than 10 ordered residues. These well-organized structures promote protein-peptide interactions wherein Gcn5 interacts mainly with the carboxyl terminal of substrates through hydrogen and van der Waal's bonds. In contrast, HAT1 binds to histone4 converting its extended, disordered amino terminal to defined, β turn conformation (Wu et al. 2012). The altered conformation of the acetylated histones shows differential interaction with DNA and other DNA-binding proteins.

2.4 Role of Post-translational Modifications in Protein Stability

Post-translational modifications alter the structure of a protein which at times leads to higher protein stability. One of the well-studied examples is hypoxia-inducible factor (HIF-1), whose levels are kept under check through its degradation. ARD1 is an acetyltransferase that adds acetyl groups to hypoxia-inducible factor (HIF-1α) and enhances its interaction with pVHL ubiquitination complex, triggered by hydroxylation of proline; protein is degraded under normal conditions. During hypoxia, p42/p44 MAPK activity induces post-translational phosphorylation of HIF-1α that leads to its stabilization and activation. Thus, stability and activation of HIF-1 involves various post-translational modifications (Hon et al. 2002; Jeong et al. 2002). Similarly, levels of p53 are controlled in unperturbed cells through its ubiquitination at C-terminal lysine residues by MDM2 whereas p53 is stabilized under stress conditions through acetylation of these lysine residues by HAT, p300. DNA damage activates protein kinases that phosphorylate p53 at serine 15 and 20. Phosphorylation at serine 18 alters the structure of amphipathic α-helix, decreasing its affinity for MDM2 (Toledo and Wahl 2006). Methylation of certain lysines adds to the stability of p53 protein. Lysine 372 methylation by SET9 stabilizes p53 (Chuikov et al. 2004) whereas methylation of lysine 370 by SMYD2 destabilizes it. p53 proline-rich domain (PRD) is also known to mediate protein stabilization through interaction with prolylisomerase (PIN1) that recruits CHK2 to phosphorylate serine 20 and consequently reduce MDM2 binding. Thus, type and site of post-translational modification of p53 is critical in determining its half-life in the cell.

E2F1, a member of E2F family of transcription factors, is another good example that is stabilized through ATM-mediated phosphorylation in response to genotoxic stress. E2F1 levels are regulated by ubiquitination-dependent proteosome degradation. However, post-translational modification by protein kinase transcription factor IIH (TFIIH) phosphorylates E2F1 that inhibits its ubiquitinization leading to its stability. E2F1 is more promptly stabilized in response to genotoxic stress through ATM and ATR kinases. Interaction of E2F1 with PCAF increases in response to DNA damage which enhances its acetylation and thus stabilization (Ianari et al. 2004).

2.5 PTMs Control Biological Functions of Proteins in the Cell

Phosphorylation and dephosphorylation of proteins is well established to alter their biological activity. A plethora of proteins including receptor proteins, enzymes and DNA-binding proteins are known to be activated upon phosphorylation (Pawson 2002). Similarly, acetylation of transcription factors, nuclear import factors and

α-tubulin alters their interactions with other partner proteins, at times serving as a scaffold protein. PTMs of proteins are reversible and induced under stress conditions.

Different types of stresses induce different post-translational modifications in p53 protein preparing it for distinct responses depending upon type of stress (Saito et al. 2003; Xu 2003). Lysine 320 acetylation inhibits apoptotic activity of p53, whereas acetylation of lysine 373 and 120 promotes activation of proapoptotic genes. Serine 46 phosphorylation is found to cause induction of genes involved in apoptosis but not the genes that mediate cell cycle arrest. Serine 6 and 9 are phosphorylated by protein kinase CK1δ and CK1ε upon genotoxic and non-genotoxic stress and mediates interaction of p53 with Smad2 which induces mesoderm-specific gene expression and integrates signalling events downstream of transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF). Mutations in serine 6 and 9 inhibit activation of p21 or cell cycle arrest, but it is able to induce expression of other p53 responsive genes suggesting distinct specificity in the functions of these modifications (Meek and Anderson 2009).

p53 is also phosphorylated at Serine 9, 15, 20 and 372 residues during G1 phase of a cell cycle, whereas S37 and S392 phosphorylation peaks during G2/M. Phosphorylation occurs selectively at S37 during S phase, and acetylation is mostly abundant at G0 (Buschmann et al. 2000). This demonstrates that these post-translational modifications are transient and dynamic and form part of normal cellular homeostasis. In another example, the C-terminus of RNA polymerase II, carrying large numbers of repeated heptapeptide consensus sequences (Tyr-Ser-Pro-Thr-Ser-Pro-Ser), besides serine and threonine residues of the enzyme, are phosphorylated, which in turn enhances the interaction of polymerase with elongation factors and associated proteins. Phosphorylation of polymerase is vital in promoter clearance and formation of elongation complex which allows RNA polymerase to move along chromatin DNA (Lee and Young 2000).

Histone acetylation plays a major role in DNA damage repair activities. Histone-specific acetyltransferase GCN5 is involved in repair of UV radiation-induced DNA damage. TFTC [TBP (TATA-binding protein)-free TAFII complex] contains GCN5 and SAP130 and preferentially acetylate nucleosomes assembled on UV-irradiated DNA. ESA1, the catalytic subunit of the NuA4 acetyltransferase complex, was found to be essential for DSB repair by both non-homologous end-joining (NHEJ) as well as the homologous recombination (HR) (Gospodinov and Herceg 2013).

2.5.1 Role of PTMs in Signal Transduction

Phosphorylation of membrane receptor proteins plays an important role in controlling signal transduction in eukaryotic cells. Membrane receptors generally have three domains: the N-terminal extra-cellular domain of polypeptide chains binds to the signal molecule, and the trans-membrane domain passes through the cellular membrane and transmits signals to the third intracellular domain. In G protein-coupled receptors, the trans-membrane domain comprises of seven α-helix polypeptide chains whereas in other proteins, this domain consists of a single α-helix region. The cytoplasmic domain generates response inside the cell through its interaction with other proteins. Cytoplasmic regions of various receptors (e.g. insulin receptor, epidermal growth factor receptor) have tyrosine kinase activity which gets activated upon binding of the ligand. Consequently, the receptor gets autophosphorylated at C-terminus that initiates the signal cascade. Receptor proteins that fail to autophosphorylate upon ligand binding associate with other cytoplasmic protein kinases that phosphorylate them. Activity of serine/threonine kinase is influenced by a number of factors such as DNA damage and/or presence of signal molecules such as cAMP, cGMP, Ca^{2+} and diacylglycerol. Apart from providing enzymatic activity, various modifications like myristoylation, farnesylation, cysteine oxidation, ubiquitination, acetylation, methylation, etc. in receptor proteins assign

scaffolding function to these proteins for the assembly of multi-protein complexes. These modifications are transient and reversible, switching on the signal cascade only when it is required.

2.5.1.1 EGFR Signalling

Epidermal growth factor receptor (EGFR) is triggered by binding of ligands like epidermal growth factor (EGF) or transforming growth factor (TGF). EGFR signalling illustrates phosphorylation of receptor tyrosine kinases at their cytoplasmic tail that enhance their interaction with proteins containing SH2 domain. The cytoplasmic tyrosine kinase SRC phosphorylates EGFR at tyrosine Tyr805 and Tyr1101 to mediate its activation (Biscardi et al. 1999). Besides tyrosine phosphorylation, EGFR can be phosphorylated at serine and threonine residues. Activation of EGFR leads to its binding with SH2 containing Grb2 and E3 ubiquitin ligase CBL. Grb2 further recruits guanine nucleotide exchange factor (GEF), which transfers GTP for GDP to Ras protein. Binding of Ras to GTP mediates conformational changes in Ras protein that enhances its interaction with kinase protein Raf (Fig. 2.3) (Marshall 1995). EGFR activation phosphorylates not only proteins but also phospholipids like phosphoionositide kinase PI3-kinase. EGFR signalling is then attenuated by rapid endocytosis (Deribe et al. 2010). Multiple protein tyrosine phosphatases are also present in a cell that dephosphorylates the tyrosines of EGFR. PTP1B, protein tyrosine phosphatase, present in endoplasmic reticulum, is involved in dephosphorylation of EGFR leading to attenuation of EGFR signalling (Haj et al. 2002).

2.5.1.2 TNFR Signalling

TNFα is a cytokine, and its binding to receptor (TNFR1) directs recruitment of adapter protein TNFR-associated death domain protein (TRADD), the E3 ligases TNFR-associated factor 2 (TRAF2), TRAF5, the inhibitor of apoptosis proteins cIAP1 and cIAP2 and protein kinase receptor–interacting protein 1 (RIP1). Ubiquitation of RIP1 activates catalytic IκB kinase (IKK) complex which phosphorylates inhibitors of NF-κB (IκB) on Ser32 and Ser36. Phosphorylation of IκB results in change in its conformation and dissociation from NF-κB. The activated NF-κB is then translocated to a nucleus where it initiates transcription of anti-apoptotic target genes. TNFR2 signalling also activates NF-κB independent of TNFR1 which stimulates non-canonical NF-κB pathway. TNFR2 induces NF-κB- inducing kinase (NIK) that stimulates IKKα which phosphorylates precursor protein p100, triggering activation of NF-κB (Naudé et al. 2011).

2.5.2 Role of PTM in DNA Damage Repair

Cells are exposed to environments where they have to endure several kinds of stress that affect stability of genome. A complex network

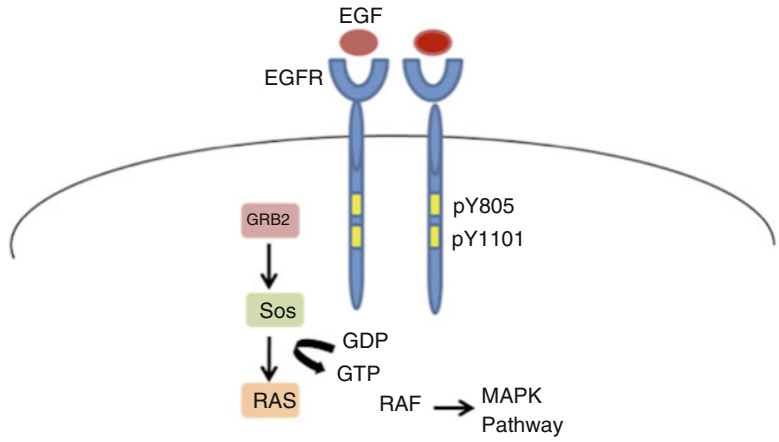

Fig. 2.3 Diagrammatic representation of EGFR Signaling pathway

of proteins is thus dedicated to repair mechanisms that are controlled by PTMs. There are various repair pathways depending on the type of stress like mismatch repair, nucleotide excision repair, homologous recombination, non-homologous end joining and trans-lesion synthesis. Histone H3-specific acetyltransferase GCN5 is involved in nucleotide exchange repair (NER) of UV radiation-induced cyclobutane primer dimers as deletion of GCN5 in yeast hampers repair process. GCN5 also participates in repair mechanisms in mammals being a component of complexes like TFTC [TBP (TATA-binding protein) – free TAFII complex] and STAGA complex. Recruitment of GCN5 to UV-damaged DNA acetylates opens up DNA, thereby increasing accessibility to repair enzymes.

Site-specific histone acetylation is crucial for double-stranded break (DSB) DNA damage repair. Mutational studies in acetyltransferases like TIP60 and MOF suggest that mutation in these proteins inhibits ionizing radiation-induced DNA damage repair. TIP60 binding to histone H3 trimethylated at lysine9 activates its acetyltransferase activity that mediates activation of ataxia telangiectasia mutated (ATM) kinase. TIP60 is phosphorylated at Tyr44 by c-Abl for its activation which is promoted by chromatin relaxation. MOF activity increases H4K16 acetylation in response to DNA damage (Kirkland and Kamakaka 2013). ATM along with ATM and Rad3-related (ATR) phosphorylates histone variant H2AX on Ser139, which recruits Mdc1. H2AX and Mdc1 together recruit other factors to the repair site.

Acetylation as well as deacetylation of histones is required at different stages of DNA repair. Non-homologous end joining (NHEJ) stimulates recruitment of acetyltransferases like p300 and CBP to DSBs that acetylates histones H3 and H4 and promotes recruitment of Ku proteins. In contrast, histone deacetylases HDAC1 and HDAC2 deacetylate H3K56 and H4K16 in response to NHEJ. Deacetylation of histones mediates chromatin compaction that prevents local transcription that may hamper repair process. Compacted chromatin prevents sliding of Ku proteins that increase repair efficiency (Gospodinov and Herceg 2013).

ATM kinase phosphorylates KAP-1, which causes its dispersion throughout nucleosomes and relaxes chromatin. Histone ubiquitinization is another regulatory element in response to DNA damage that is required for recruitment of BRCA1 and 53BP1. MDC1 recruits RNF8 E3 ubiquitin ligase to repair foci. RNF2 promotes mono-ubiquitination of γ-H2AX which is required for addition of ubiquitin residues by RNF8 and RNF168. Ubiquitylated histones are bound by RNF80 that forms a bridge between BRCA1 and histones (Mailand et al. 2007; Huen et al. 2007; Kolas et al. 2007).

Methylation of histones also plays an important role in DNA damage repair as dimethylation of H4K20 is required for accumulation of 53BP1 in repair foci. Dimethylation of H4K20 increases in response to DNA damage, and the process is catalyzed by Suv4-20 h methyltransferase. Conversion of dimethylation to monomethylation increases sensitivity to DNA damage (Schotta et al. 2008).

2.5.3 Modulation of Gene Expression Due to PTMs of Histone and Non-histone Proteins

Post-translational modifications in histones provide basis for modulation of chromatin structure. E2F and its pocket proteins add or remove histone modifications to mediate gene activation or repression. E2F family comprises of eight members, some of which are considered to be activators while E2F4 and E2F5 form repressive complexes. E2F4 and E2F5 are present in cytoplasm, and its interaction with hypophosphorylated Rb induces its transport into nucleus (Grandinetti and David 2008). E2F4 recruits Sin3B/HDAC co-repressor complex as the cell enters G_0 phase causing methylation of histone H3 at lysine 9 and lysine 27, markers of repression (Fig. 2.4) (Blais and Dynlacht 2007). During a late G1phase, activated E2Fs bind to promoters of proliferative genes. Recruitment of E2F to

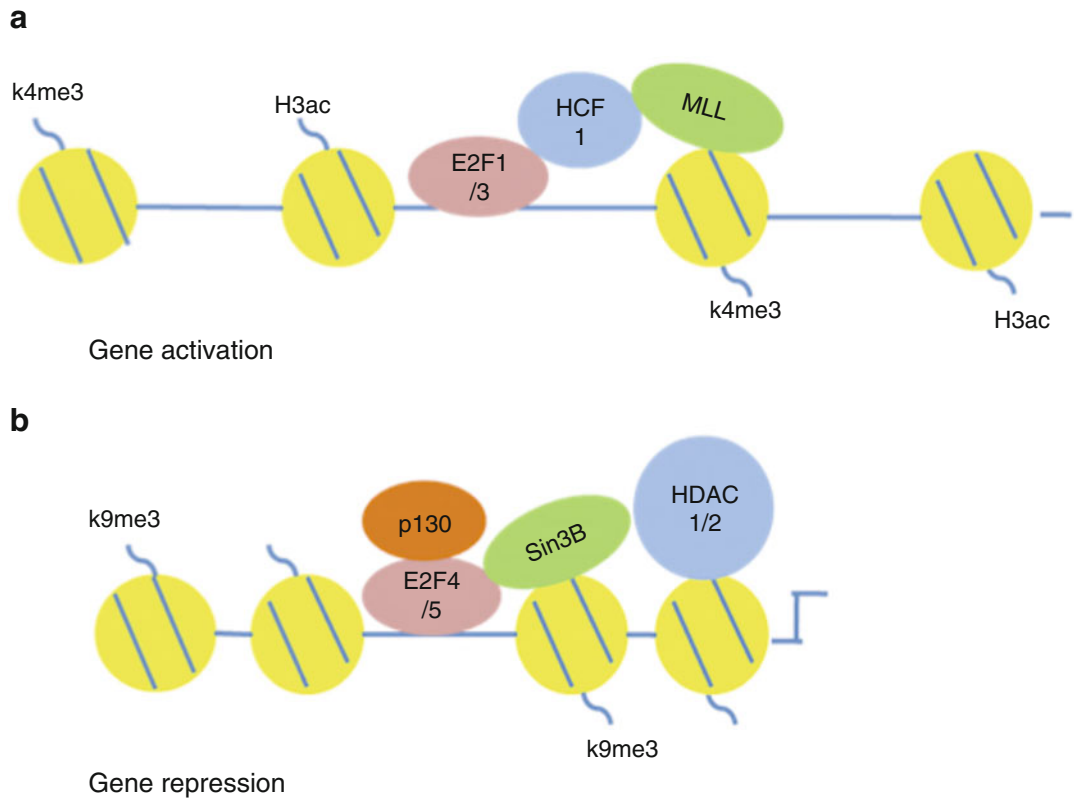

Fig. 2.4 Model describing certain histone modifications that occur during gene activation and repression. Various HATs are recruited on the promoter of genes that mediate histone acetylation leading to opening of chromatin and gene activation. On the contrary, HDACs mediate deacetylation and compact chromatin structure

promoters facilitates recruitment of histone acetyltransferases like p300/CBP and PCAF/GCN5 that promote acetylation of histones leading to gene activation. In addition, histone H4 is trimethylated at lysine4 by histone methyltransferases of mixed-lineage leukemia (MLL) and SET families. However, histones bearing acetylation marks are also recognized by bromodomain family of proteins. Bromodomain is present in many transcription factors like HATs and BET nuclear factors and stabilizes interaction of these factors with acetylated histones. Testis-specific bromodomain-containing Brdt recognizes histone H4 acetylated specifically at K5/K8. Thus, acetylation and methylation of histones differentially regulate gene expression in eukaryotes by altering the binding of transcription factors to the promoter.

In developing or differentiated cells, acetylated histone H3 and H4 near transcriptional start site (TSS) is correlated with gene activation. For example, stimulation of monocytes requires acetylation of histones downstream of IL-*1β* TSS and H4 acetylation upstream of IL-*1β* (Liang et al. 2006). Differentiation of naïve T cells into mature Th1 or Th2 cells is another good example of roles of histone modifications across IFN-γ and IL-4 loci.

In contrast to acetylation and deacetylation of histones, little is known about other modifications in histones. Phosphorylation of serine 10 in histone H3 is correlated with gene activation that adds negative charge to histones reducing its affinity to bind DNA (Thomson et al. 1999; Khorasanizadeh 2004). Mutation of serine 10 of histone H2A caused spurious chromosome condensation. Further studies revealed the role of phosphorylation of serine 10 of H2A in chromosome condensation.

2.6 Conclusion and Future Perspectives

From the foregoing examples, it is evident that post-translational modifications in proteins such as acetylation, methylation, hydroxylation and sulfation are critical in regulating cellular homeostasis. Numerous studies have established that post-translational modifications play an important role in mediating signal transduction in cells. Moreover, acetylation and methylation regulate gene expression in cells through controlled chromatin structure. These evidences suggest that PTM in proteins is an inevitable process required for normal cellular functioning. Although a few novel PTMs have been recognized in certain proteins, the function of these modifications is not clear and would require *in vivo* studies to decipher them. PTMs can be linked to neoplasmic transformation as the major functions like gene expression and cellular signalling that are disrupted in cancer are controlled by modulation of protein structure and activity due to post-translational modifications. It is also believed that regulating the activity of some of these modifying enzymes may pave the way for treatment of cancer and other chronic diseases. Inhibitors of HDACs are already being studied extensively for their use as anticancer drugs. Future studies need to be directed in understanding the role of the PTMs in other chronic diseases and the potential use of the modifying enzymes/proteins as drug targets.

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Small Molecule Osmolytes Can Modulate Proteostasis

3

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Contents

Abstract

Organisms are subjected to several harsh extreme environmental conditions and stresses that normally hamper growth and their physiological function. In response, nature has developed certain mechanisms to counteract these extreme stress conditions and one such mechanism is the accumulation of certain small molecular weight organic solutes called osmolytes. These osmolytes protect intracellular proteins and other macromolecules against the deleterious effects induced by these stress conditions. In addition to these protecting roles of these solutes against stress, they have also been shown to correct protein misfolding, relieve aggregation and amyloidogenic progression in various proteopathies

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related proteins. The present chapter discusses the significant advances made in the field of mechanisms of the osmolytes-induced protein folding/stabilization. The chapter has been designed so as to include all aspects of osmolytes actions on proteins stability and functions. Later sections also highlight the possible use of these organic osmolytes as pharmaceutical drugs in preventing aggregation/amyloidogenesis related human diseases.

Keywords
Osmolytes • Chemical chaperones • Protein folding • Folding pathway • Protein misfolding • Protein aggregation

3.1 Introduction

Protein folding is highly complex physicochemical phenomenon occurring within fractions of a second through which the amino acids sequence of the newly synthesized polypeptide chain arrange themselves from an unorganized unfolded state to a highly organized unique native structure adopting a final functional conformation. Thus the process of protein folding must occur with great fidelity as all other cellular life processes depend on the final product of this particular event. It is well established that not only the amino acid sequence (as invoked by Anfinsen) (Anfinsen 1973) *par se*, is important in making the unfolded proteins *en-route* to the native functional state, but also the solvent environment wherein protein folding takes place. The protein folding solvent environment mainly consists of salts, small molecule compounds, metabolites, molecular chaperones and other chemical species that in fact aid in the folding process. Therefore, a subtle change in the composition of the protein folding environment will alter the protein folding process. The importance of the solvent environment in protein folding came from the very fact that various disease causing proteopathies can be reversed back by manipulating the solvent environment of the malfolded proteins. It will not be surprising that more than 30 % of the processed final end products of this folding reaction consists of malfolded conformation and thus are degraded via proteosomal pathway. This indicates the complexities of the process which could also generate misfolded non-native product having great propensities to form protein aggregates (Schubert et al. 2000), when the protein degradative machinery could not cope up with the situations (large misfolding processes). Hostile environmental stresses are the basic cause of such large misfolding conditions. Since cells commonly encounter extreme environmental fluctuations, it is crucial that they should equip themselves with strategies to circumvent the hostile environmental situations.

Organisms (plants, animals and microorganisms) are known to adapt to a number of hostile abiotic and environmental stresses which would normally disrupt the macromolecules structure and function, thereby hampering life giving cellular processes. These hostile conditions involve stresses such as extremes of temperature, pH, cellular dehydration, desiccation, high extracellular salt and even the presence of high levels of denaturing concentrations of urea inside cells. One mechanism of adaptation which protects cellular components against these denaturing stress conditions involves accumulation of small organic molecules known as osmolytes within the cells (Yancey 2003, 2004; Yancey et al. 1982). Osmolytes are naturally occurring small molecular weight compounds accumulated intracellularly to protect organisms from these stresses. They may accumulated in the intracellular environment at relatively high concentrations (upto several millimolar concentration at certain tissues depending on the stress types) (Bolen and Baskakov 2001; Burg 1995). The term "chemical chaperones" was chosen to reflect that although these compounds possess properties akin to that of molecular chaperones, they are not protein molecules that facilitate protein folding and contribute to acquire thermotolerance. The evolutionary advantages of the accumulation of such organic osmolytes during hostile environmental stresses are as follows: (i) They provide stabilization of cellular proteins and other macromolecules against the denaturing environmental stress without significantly affecting their functional activity (Somero 1986; Yancey et al. 1982; Yancey and Somero 1980a, b).

(ii) Their stabilizing effects are not confined to a particular type of protein and thus prevent large change in proteome to satisfy the changing need of the cellular environment.

3.2 Classification

Organic osmolytes are highly diverse in nature, and their distribution in the various organisms varies significantly. These osmolytes fall into different types of compounds and differ both chemically and functionally (see Table 3.1). Chemically, these osmolytes can be grouped into three major classes (as in Table 1 in Singh et al. 2011): polyols (e.g., mannitol, glycerol, sorbitol, inositol, pinitol, sugar and sugar derivatives), free amino acids (e.g., glycine, alanine, proline) and their derivatives (taurine, octopine, β-alanine), and methyl ammonium compounds (e.g., trimethylamine-N-oxide (TMAO), glycerophosphocholine (GPC), glycine betaine, and sarcosine) (Welch and Brown 1996). This classification has been solely based on the chemical property of the compounds and presents no functional attribution to the protein stability and functionality. Further distinction has been made according to the manner in which the functional activity of a protein is maintained in the cells by particular type of osmolytes within this three chemical classes mentioned above. This has, therefore, led to the classification of organic osmolytes either as "compatible" or "counteracting" (Bowlus and Somero 1979; Yancey et al. 1982). Compatible osmolytes are those that stabilize proteins without substantively affecting the functional activity parameters of a protein (Bowlus and Somero 1979; Pollard and Wynjones 1979). Representatives of this class include certain amino acids, their derivatives, and polyols. Major stress conditions that these compatible osmolytes protect against include cellular dehydration, high-salt environments, extremes of temperature, etc. (Yancey et al. 1982). On the other hand, counteracting osmolytes (which consist of the methylamine class of osmolytes in major) are believed to have special ability to protect intracellular proteins against the inactivating effects of urea and salts on proteins (Lin and Timasheff 1994). Furthermore, in contrast to compatible osmolytes, which do not largely affect the functional activities of proteins, counteracting osmolytes are known to cause changes in protein functions that are the opposite of the effects induced by that of urea and salts on proteins (Somero 1986).

More recently, based on structure-function relationship of various stabilizing osmolytes, Ahmad and co-workers (Jamal et al. 2009) put forward another classification of organic osmo-

Table 3.1 Classfication of osmolytes based on chemical, function and structure-function relation

	1. Stabilizing osmolytes	
I. Chemical classification		
Amino acids & derivatives	**Polyols and sugars**	**Methylammonium salts**
Proline, phenylalanine, valine, leucine, isoleucine, serine, glutamine, arginine, lysine, glycine, aspartate, β-alanine, ectoine, taurine, hypotaurine, thiotaurine	Glycerol, sorbitol, mannitol, pinitol, inositol, glucose, fructose, sucrose, raffinose, stachyose, trehalose, mammoslyglycerate, glucosylglycerate	Glycine betaine, L-carnitine, glycerophosphorylcholine, choline, creatine, trimethylamine, N-oxide, N-methyltaurine, sarcosine
II. Functional classification		
Compatible osmolytes	**Counteracting osmolytes**	
Sugars, amino acids, polyols & their derivatives	Methylammonium salts	
III. Structure-function relations		
Class I	**Class II**	**Class III**
Amino acids, polyols and their derivatives	Methylammonium salts	Sugars
	2. Destabilizing osmolytes	
	Lysine, arginine, histidine, urea	

lytes. This group has distributed all stabilizing osmolytes into three broad classes. Included in Class I are polyhydric alcohols and amino acids and their derivatives that have no significant effects on both the protein stability in terms of Gibbs free energy change at 25 °C ($\Delta G_D°$) and the catalytic efficiency (k_{cat}). Methylamines compounds that increase both $\Delta G_D°$ and k_{cat}, but decrease the Michaelis-Menton constant (K_m) are included in Class II. Sugars that increase $\Delta G_D°$, but decrease both K_m and k_{cat} belong to class III.

It is also known that not all osmolytes accumulated by cells are stabilizing in nature but a few osmolytes are destabilizing or denaturing to the macromolecules. These osmolytes principally include arginine, lysine and histidine. In addition to these free amino acids, urea is also a strong osmoprotectant accumulated specially in mammalian kidney cells (for details, see "*Methylamine offsets harmful effects of urea in mammalian renal cells*"). A list of major classes of osmolytes accumulated by organisms, and representative examples are provided in Table 3.1.

3.3 Compatibility Paradigms of Osmolytes

3.3.1 Functional Compatibility

Several lines of evidences suggest that these osmolytes do not significantly perturb cellular functions and enzyme activities under physiological conditions. Studies from two different laboratories (Yancey and co-workers, Timasheff and co-workers) on several enzymes came to the fact that specific activities of the enzymes remain unaltered in the presence of various osmolytes. Further investigations on the effects of osmolytes on the kinetic parameters revealed that both K_m and k_{cat} of proteins are not significantly altered in the presence of the osmolytes. With a few exceptions, polyol and amino acid osmolytes are compatible with enzyme function (Jamal et al. 2009). However, glycerol is not found to be a universal compatible osmolyte as it alters the activities of some enzymes (Bolen and Fisher 1969; Myers and Jakoby 1973; Stamatakis et al. 1988). In contrast to the effects of amino acids and polyols, methylamine compounds are found to decrease K_m and increase k_{cat} thereby increasing the specific activity of enzymes. It is really hard to understand why methylamine class of osmolytes are accumulated in high concentration as they are not compatible with the functions of enzymes tested. However, they also appear to be compatible in the presence of another osmolyte urea. Therefore, it seems likely that almost all solutes accumulated under stress conditions are necessarily compatible with enzyme functions. Most recently, it has also been shown that osmolytes do not really interact with the hydrophobic groups present in proteins (Athawale et al. 2005). Hydrophobic groups are mainly responsible to collapse the unfolded protein and to en route folding to proper native structure. It has been suggested that the neutrality of osmolytes towards hydrophobic interaction (a major force in protein folding) might also be the origin of functional compatibility of enzymes.

3.3.2 Thermodynamic Compatibility

In addition to the functional compatibility, several osmolytes (polyols, amino acids and derivatives), although they increase T_m (melting temperature) do not alter the $\Delta G_D°$ (Gibbs free energy change at 25 °C) of proteins significantly indicating that the osmolytes are thermodynamically compatible at physiological conditions. However, it is not still known why osmolytes affect only the T_m but not in the $\Delta G_D°$ of proteins at physiological conditions. It was argued that the main reason for not perturbing the $\Delta G_D°$ of proteins by osmolytes at physiological pH and temperature is due to the fact that there is perfect enthalpy-entropy compensation in the presence of osmolytes or, in other words, there is perfect balance of preferential exclusion (stabilizing force) and preferential binding (destabilizing

force) of osmolytes to the proteins (Haque et al. 2005a, b, 2006; Jamal et al. 2009). Thermodynamic compatibility strictly confines to the physiological condition. However at other solvent conditions like low pH or other denaturing conditions, non-perturbing nature of osmolytes against ΔG_D° of proteins disappeared suggesting that the protein thermodynamic compatibility has not been evolved at other solvent conditions except the physiological conditions.

3.3.3 Structural Compatibility

The compatibility paradigm of osmolytes holds true for proteins structure too. Structural non-perturbing natures of osmolytes come from three basic studies (Spectroscopy, X-ray crystallography and molecular simulation studies). Using circular dichroism spectroscopy, Ahmad and co-workers have shown that for many osmolytes, the secondary and tertiary structures of several proteins remain unaltered due to the presence of osmolytes (Haque et al. 2005a, b; Poddar et al. 2008; Singh et al. 2005). Size exclusion chromatography results also revealed that osmolytes have no effects on the dimensions of the protein native fold (Baskakov and Bolen 1998; Baskakov et al. 1998; Qu et al. 1998). From the studies on co-crystallization of TMAO and RNase-A fragment, it was concluded that the structure of RNase-A is not affected by TMAO (Ratnaparkhi and Varadarajan 2001). Various other spectroscopic studies further strengthen this structural compatibility paradigm. This conclusion is also supported by the two-dimensional NMR studies that measure exchange rates of individual labile protons. It has been observed that glycine (up to 2.0 M concentrations) has negligible effect on the intrinsic quality and nature of the NMR spectra of proteins (Foord and Leatherbarrow 1998). Simulation studies (Athawale et al. 2005) have also proved that osmolytes simply increase the water-water H-bond strength, shortens the H-bond length and therefore increase the water of hydration but this does not affect the hydrophobic interactions.

3.4 Osmolytes Protect Macromolecules from Stress by Increasing T_m

Osmolytes generally stabilize proteins, not by interacting with them directly but by altering the solvent properties of the surrounding water and hence the protein–solvent interactions. Their effect seems to be general for all proteins. The thermodynamic effect of osmolytes on protein stability can be understood from the increase in T_m (melting temperature) or C_m (melting concentration) of proteins in their presence. A list of osmolytes along with the thermodynamic parameters obtained in the presence of osmolytes is listed in Table 3.2. Although all osmolytes are known to enhance T_m of proteins, there are subtle variations in the stabilization due to difference in the chemical nature of the osmolytes. For example, polyols increase T_m not only in a concentration dependent manner (Haque et al. 2005a, b, 2006) but also as a function of the length and number of OH groups of the polyhydric alcohol (Gerlsma 1968). The larger the number of OH groups the higher is the T_m of the protein raised. Similar to polyols, the effect of sugar osmolytes not only show a linear function of concentration but also largely depend on the size of the sugars (Poddar et al. 2008). Amino acid osmolytes also increase T_m in a concentration dependent manner but the effect on T_m due to variation of chemical nature seems indifferent (Singh et al. 2011).

Table 3.2 Thermodynamic parameters of Lysozyme at pH 7.0 and 25 °C

[Osmolytes]	T_m	ΔH_m	ΔG_D^o
Control	86.0	129.7	13.1
Xylitol[a] 1.0 M	90.0	116.8	14.2
Adonitol[a] 1.0 M	89.3	119.7	13.8
Mannitol[a] 1.0 M	90.0	123.8	14.1
TMAO[b] 1.0 M	91.9	144.8	15.5
Sarcosine[b] 1.0 M	90.0	142.6	15.5
Betaine[b] 1.0 M	89.1	140.9	15.1

The units of ΔG_D°, ΔH_m and T_m are kcal mol^{-1}, kcal mol^{-1} and °C, respectively

[a] & [b] were taken from Haque et al. (2005a, b) and Singh et al. (2007a, b), respectively

Methylamines class of osmolytes also enhances T_m of proteins in a concentration dependent manner (Singh et al. 2005, 2007a). However, the osmolytes are found to have a tendency to destabilize proteins (or decrease T_m) at high concentrations due to its propensity to bind to the exposed hydrophobic groups in the denatured state (Santoro et al. 1992). In contrast to the effect shown by osmolytes on T_m of protein, the effect on ΔG_D° is indeed clumsy. Although the polyol class of osmolytes increases T_m or C_m, they are found not to alter ΔG_D° of protein. For example, almost all polyol osmolytes (sorbitol, xylitol, mannitol, glycerol, adonitol) are found to have no significant effect on protein stability (in terms of ΔG_D°) at physiological conditions (Haque et al. 2005a). Authors argued that this might be the reason why the protein turnover is not perturbed by the presence of osmolytes at physiological pH and temperature. However, ΔG_D° of protein is found to be increased by polyol osmolytes at destabilizing conditions suggesting that osmolytes might act on the unstable proteins (Haque et al. 2005a, 2006). On the other hand, methylamines are found to have increased ΔG_D° of protein at physiological conditions (Singh et al. 2005). This might be the reason why these osmolytes are especially accumulated to oppose the high concentration of urea in cells.

The protein stabilization in terms of ΔG_D° has been reported to depend on two factors namely, ΔH_D° (ΔH_D, the denaturational enthalpy change at 25 °C) and ΔS_D° (ΔS_D, the denaturational entropy change at 25 °C). Estimation of enthalpy and entropy contributions to ΔG_D° obtained in the presence of polyol osmolytes using appropriate thermodynamic relations revealed that there is a perfect enthalpy-entropy compensation of proteins in the presence of all polyol osmolytes at pH 7.0 (Haque et al. 2005b, 2006). Thus, thermodynamically the non-perturbing nature of compatible osmolytes on ΔG_D° is primarily due to the perfect balance between enthalpy and entropy. In case of methylamines, the balance between enthalpy and entropy gets disturbed even at physiological conditions (Singh et al. 2005). Singh et al. (2005) have shown that ΔH_D° and ΔS_D° of lysozyme, RNase-A, and α-LA in presence of TMAO at physiological pH and temperature are greater than zero i.e. protein stabilization by TMAO is enthalpically unfavourable and entropically favourable. This unfavourable enthalpy change outweighs the favourable entropy change and hence yields an unfavourable free energy change ($\Delta G_D^\circ > 0$). Thus stabilization of all proteins by TMAO is enthalpically controlled at physiological pH.

The effect of some osmolytes on ΔG_D° is, in fact, denaturing at some solvent conditions. TMAO, although shows stabilizing effect on proteins near neutral pH, destabilize proteins at lower pH values (pH 5.0 and lower) (Singh et al. 2005). Another methylamine osmolyte, glycine betaine was also found to destabilize or lose its stabilization power at lower pH values (pH 5.0 and below) while it still stands as a strong protein stabilizer near neutral pH (Singh et al. 2009).

3.5 Osmophobic Effect Forces Protein Folding

On the basis of transfer free energy measurements of amino acid side chains and peptide backbone from water to osmolyte solutions, Bolen and co-workers have demonstrated that osmolyte-induced folding of proteins mainly originates from the predominant unfavourable interaction of osmolytes with the protein peptide backbone (Liu and Bolen 1995; Qu et al. 1998; Wang and Bolen 1997) whereas the side chain interactions contribute weakly. The unfavourable interactions between a solvent component (i.e. the osmolyte solution) and a protein functional group (the peptide backbone) are traditionally classified as solvophobic, and this unfavourable interaction has been termed as the osmophobic effect (Bolen and Baskakov 2001). Transferring a native protein to osmolyte solution increases the chemical potential, i.e. destabilizing the native state. The reason why osmolyte stabilizes the proteins against denaturation is that they destabi-

lize the unfolded state due to unfavourable interaction with the peptide backbone much more than they destabilize the native state (Bolen and Baskakov 2001).

In a similar development, based on the preferential interaction measurements between the osmolytes and proteins, Timasheff and his group are able to demonstrate that the denatured proteins are preferentially more hydrated than the native protein indicating that the destabilization of the denatured state is due to overwhelming preferential hydration and hence the origin of osmolytes induced protein folding (Timasheff 2002a, b). In fact, the native state preferential hydration is not perturbed by osmolytes. However, osmolytes may have preferential binding affinity in addition to the preferential hydration ability on the proteins. Therefore, what effect osmolytes will have on the denatured/unfolded protein is determined by the balance of the binding and/or exclusion of the co-solute from the denatured protein surface. In terms of thermodynamics of protein folding or stabilization, preferential exclusion of osmolyte solutes from the protein surface shifts the protein thermodynamic equilibrium, N state ↔ D state toward the native state and preferential binding shifts in equilibrium towards the denatured state. Therefore, effect of an osmolyte on the denaturation equilibrium is defined by the balance of preferential binding and exclusion from the protein surface. In addition to this concept of preferential hydration and osmophobic theory, there are other models that are commonly used to explain the protein-osmolyte interaction.

Steric Exclusion effect: The 'excluded volume' of a molecule is the volume that is inaccessible to other molecules in the system as a result of the presence of the first molecule. Excluded-volume effects result from the increase in steric exclusion in solute-solvent mixtures relative to pure solvent (Berg 1990; Knoll and Hermans 1983; Minton 1997). The phenomenon of steric exclusion was first proposed by Kauzmann (Kauzmann et al. 1949) which is solely based on the size difference of water molecules and to that of the co-solutes, osmolytes here in this case. It is assumed that the co-solutes cannot penetrate the protein structure, and hence an impenetrable shell is thus formed around the protein molecule. Water molecules being much smaller than the co-solute size particles, they are able to penetrate this shell, and hence a water-rich zone is formed around the protein molecule. This is the origin of preferential hydration of proteins (in water co-solutes system which leads to the stabilization/folding of protein molecules). Preferential exclusion, another interrelated phenomenon, is the basis of osmolytes induced proteins stabilization/folding. It has been shown that it is this exclusion of stabilizing co-solutes from the protein vicinity that lead to stabilization/folding (Arakawa and Timasheff 1982, 1985).

Kirkwood Buff Theory: Rosgen and co-workers (Harries and Rosgen 2008; Rösgen 2009) developed a mathematical model to determine hydration and osmolyte solvation (osmolation) of peptide backbone for all the classes of osmolytes using Kirkwood–Buff theory. The model helped us to understand that the major solvation effects on protein side-chains originate from the osmolytes, and that the size of the side-chain of the proteins determines the extent of hydration. In different osmolyte solutions, the peptide backbone displays much variable hydration as compared to the side chains that could led them to group osmolytes based on their peptide unit solvation behaviour. Although methylamine osmolytes, such as TMAO, sarcosine and betaine, show very little change in the hydration of the peptide unit upon transfer from water to 1 M osmolyte, the osmolation is large and negative. These observations indicate that the all methylamines are strongly excluded from the peptide unit. Similar is the case for the osmolytes such as proline, glycerol and sorbitol. However, due to the presence of these osmolytes, a large amount of water is also excluded from the peptide unit. Contrary to the above- mentioned cases, the saccharides, sucrose and trehalose favourably interact with the peptide unit and hence the peptide unit becomes excessively hydrated. Sucrose is reported to be even more enriched around peptide groups (Auton et al. 2008).

Scaled particle theory (SPT): SPT is a statistical mechanical approach often applied for predicting the behaviour of solvent-based system, such as stability of a protein, in a co-solutes formulated solution (Davis-Searles et al. 2001; Lebowitz et al. 1965). Such analysis assumes that the solution constitutes of a mixture of hard spheres of various radii as proposed by Reiss and co-workers (Reiss et al. 1959). Successful placement of a hard sphere of the protein molecules into the solution containing various sizes of co-solute particles denotes cavity formation. The cavity formed equals to the radius of the inserted particle. The chemical potential of a hardcore particle is equal to the mechanical work needed to inflate the cavity of radius 'r'. SPT will therefore predict the change in the Gibbs free energy of the cavity formation in the system in terms of the work done in forming a cavity large enough to accommodate the protein (Reiss et al. 1960). Calculation of ΔG_D^o for the protein during the transition between the native and denatured states requires the hard sphere radius in both the native and denatured states. According to SPT, the stability of proteins in the presence of osmolytes is due to the increase in the free energy of protein-solvent interface formation (O'Connor et al. 2007). Ahmad and co-workers have earlier recently shown that the SPT can be fairly used to explain the thermodynamics of protein-osmolyte interactions (Poddar et al. 2010).

3.6 Osmolytes Can Reshape the Protein Folding Pathway

Recently, in addition to the effect of osmolytes on the thermodynamic equilibrium, it has also been shown that osmolytes also effects the intermediate states of a protein folding reaction. Molten globule states (or A states) are essential intermediates in the protein folding reaction. It has been shown earlier on cytochrome-c that osmolyte like sugar-induced molten globule state formation (Davis-Searles et al. 1998). It is also known that these osmolytes can also stabilize the conformation of the molten globule states of proteins. In another study, it was shown that molten globule states are more preferentially stabilized than the unfolded states of glutaminyl tRNA synthetase (Mandal et al. 2003). These evidences clearly indicate that osmolytes also influence the protein folding pathway by acting on the intermediates rather than the unfolded states of a protein. Recently, it was also shown that sarcosine and TMAO increases the structural homogeneity of an early intermediate ensemble in the barstar-folding pathway (Pradeep and Udgaonkar 2004). Both osmolytes also alter the structure of intermediate ensemble but do so differentially; the fluorescence and circular dichroism properties of intermediate ensemble differ in the presence of the different osmolytes. Because these properties also differ from those of the unfolded form in refolding conditions, different burst phase changes in the optical signals are seen for folding in the presence of the different osmolytes. An analysis of the urea dependence of the burst phase changes in fluorescence and circular dichroism demonstrates that the formation of intermediate ensemble is itself a multistep process during folding and that the two osmolytes act by stabilizing differentially the different structural components present in the intermediate ensemble (Pradeep and Udgaonkar 2004). Thus, osmolytes can alter the basic nature of a protein folding pathway by discriminating, through differential stabilization, between different members of an early intermediate ensemble, and in doing so, they thereby appear to channel folding along one route when many routes are available.

Osmolytes are also found to affect the kinetics of protein folding and hence the rate of the protein folding reaction. Osmolyte-induced protein stabilization/folding has been observed due to increased folding rates (Jacob et al. 1997; Plaxco and Baker 1998), presumably by facilitating the condensation of polypeptide chains into the semi-compact transition state, which is the rate-limiting step in a protein folding reaction. Some groups argue that since osmolytes are viscogenic in nature, it could retard the diffusion process of polypeptides through the solvent and therefore reduce the rate of folding (Chrunyk and Matthews

1990; Goldberg and Baldwin 1998; Jacob et al. 1997; Jacob and Schmid 1999; Plaxco and Baker 1998; Waldburger et al. 1996). Such retardation in the folding rate is clearly seen in case of cold-shock protein calpastatin B. It has been shown that there is a distinct retardation of the folding rate at increased viscosity by osmolytes or other viscogenic agents, indicating that displacement of solvent is an important determinant of the kinetics of the rapidly folding protein (Chrunyk and Matthews 1990; Goldberg and Baldwin 1998; Jacob et al. 1997; Jacob and Schmid 1999; Ladurner and Fersht 1999; Plaxco and Baker 1998; Waldburger et al. 1996). However, chymotrypsin inhibitor 2 is observed to be largely unaltered by solvent viscosity. In chymotrypsin inhibitor 2, it was shown that there is a steady increase in folding rate upon addition of sucrose (Ladurner and Fersht 1999). The lack of diffusive character in chymotrypsin inhibitor 2 indicates that this protein crosses the diffusive barrier without significant displacement of osmolyte molecules (Ladurner and Fersht 1999).

3.7 Osmolytes Attenuate Native State Internal Dynamics

It is known that osmolytes do not largely affect the native state conformations and hence compatible with protein structure. It may, however, be noted that the functions of every protein existing in nature are attributed not only to their native state conformation but also to the internal dynamic characteristics they posses. Recent advances indicate that the native state of a protein consists of different sub-states that have subtle difference in the free energy (Frauenfelder et al. 1979). On the other hand, native proteins breathe to maintain an internal dynamism. A very intimate association exists between a protein functionality and stability with that of the internal dynamics (Liu and Bolen 1995). Till date, much of our understanding on the osmolytes induced protein folding and stabilization have been explained mainly through the principles of unfavourable osmophobic interactions between the protein peptide backbones and the solutes (i.e. osmolytes) and preferential exclusion mechanisms. Very fewer aspects on the effects of these osmolytes on the internal dynamics of the folded native ensembles are explored and hence remain unavailable.

According to the excluded volume model, osmolytes limit the conformational freedom of proteins by driving them to their most compact native state conformation (catalytically the most efficient form). The decrease in conformational freedom results due to the steric repulsions between the protein and the osmolyte. The latter model assumes that the native state of a protein consists of a number of inter-converting high (most compact) and low (less compact) activity state ensembles and also demonstrated that the presence of osmolytes in the protein solution shifts the native conformational equilibria towards the most compact protein species within native state ensembles (Baskakov et al. 1999; Yancey and Somero 1980a, b). Tryptophan phosphorescence is an excellent tool to observe any change in protein structural flexibility. Using this spectroscopy technique, phosphorescence lifetime in the presence and absence of osmolytes were measured. From the measurement in the presence of polyol osmolytes such as trehalose, xylitol and sucrose, across a wide range of temperature, Strambini and co-workers (Cioni et al. 2005; Gonnelli and Strambini 1995, 2005) have shown that there is a sharp distinction between proteins with a compact globular fold and internally hydrated proteins in terms of perturbation by internal fluctuations by osmolytes. From the modulation of chromophore ($_T$) and bimolecular rate constant (kq) of the protein azurin in the presence of trehalose, xylitol and sucrose across a wide temperature range, it was observed that osmolytes attenuate the structural fluctuations principally when proteins are internally hydrated or thermally expanded. The group also investigated in other proteins (alcohol dehydrogenase, apoazurin, glyceraldehydes-3-phosphate dehydrogenase and alkaline phosphatase) and demonstrated that TMAO makes no significant change in the flexibility of these proteins in a temperature range between −10 °C and to their melting temperature

(Gonnelli and Strambini 2001). Hence the attenuation of internal structural fluctuation in the native state, on the other hand, is not a general response to the osmolyte. In another study, Kim et al. (2003) have shown that sucrose shifts the conformational equilibrium within native state ensembles of the proteins toward compact conformations with more ordered structures. Favouring the most compact sub-states within the inter-converting native state ensembles could explain how osmolytes alter the catalytic efficiencies of enzymes (Fields et al. 2001). It thus appears that in addition to inhibiting protein denaturation and enhancing protein stabilization, another fundamental property of osmolytes is to influence the conformational internal dynamics of the native state.

Osmolytes increase the thermodynamic conformational stability of proteins, shifting the equilibrium between native and denatured states favouring the native state. However, their effects on conformational equilibrium within native-state ensembles of proteins remain controversial (Kim et al. 2003). The effects of osmolytes on the internal dynamics and local flexibilities of protein conformation are not properly understood, but it is now known that different osmolyte classes may have different consequences on the ensemble nature of the native state (Jamal et al. 2009). More systematic approaches and studies are needed to be undertaken to understand clearly the role of these organic solutes on the internal dynamics and proteins substates.

3.8 Osmolytes Protect Macromolecules from Stresses

3.8.1 Osmolyte Protect Macromolecules from High Temperature

High temperature constitutes one of the major stresses for organisms living in hydrothermal vents and geysers. Carbohydrate solutes are the major osmolytes accumulated in response to such extreme temperature regime in many organisms. Sorbitol and trehalose are common solutes under high temperature in insects (Wolfe 2000) and yeast (Balakumar and Arasaratnam 2012), respectively. Di-*myo*-inositol phosphate is dominated in the hyperthermophilic archaeon with thermal stress. It has also been shown that di-myo-inositol phosphate is a potent protein thermostabilizer (Martin et al. 1999). Proline serves as a universal thermal protector in many common plants. Numerous studies demonstrated that most osmolytes increase protein thermal stability in vitro (Yancey 1994). Methylamines (Yancey 1994) and trehalose (Sola-Penna and Meyer-Fernandes 1998) are often most effective.

3.8.2 Methylamine Osmolytes Protect Macromolecules from High Pressure

It is known that hydrostatic pressure can perturb protein structure and function. Yet many organisms survive in the deep sea, where high hydrostatic pressure can perturb proteins and other macromolecules (Siebenaller and Somero 1989). To circumvent the effect of pressure, these organisms appear to accumulate many osmolytic compounds. For instance, in deep-sea teleosts and other animals, TMAO is accumulated and its level reaches up to 300 mmol kg^{-1} while it is <100 mmol kg^{-1} wet wt in shallow marine animals (Yancey 2001). In vitro enzyme activity measurement discovered that TMAO offsets pressure-inhibited stability of several lactate dehydrogenase (LDH) homologues (Yancey and Siebenaller 1999) and polymerization of actin (Yancey 2001). However, glycine could not afford to reverse the pressure-induced inhibition (Yancey 2001). TMAO also offset pressure-induced increases in enzyme K_m values of LDH (Yancey 2001). However, not all deep-sea animals accumulate TMAO perhaps they lack TMAO synthesis pathways but are found to have high levels of rare osmolytes, including *scyllo-inositol* in echinoderms and hypotaurine and methyltaurine in vestimentiferans (Yin et al. 2000).

3.8.3 Methylamine Offsets Harmful Effects of Urea in Mammalian Renal Cells

Urea is a chaotropic agent that disrupts hydrophobic interactions responsible for the globular structure of proteins (Nozaki and Tanford, 1963; Yancey and Somero 1979, 1980a, b). This loss of structure influences maximal velocity (V_{max}) and K_m (Yancey and Somero 1979, 1980a, b) and alters the T_m of proteins (Nozaki and Tanford, 1963; Singh et al. 2008). Urea also binds to proteins, dehydrating their exposed surfaces and promoting denaturation (Creighton 1991; Wu and Wang, 1999; Zou et al. 1998). Intracellularly high urea concentration also lead to post-translational modification of proteins by either carbamoylation or carbonylation (Kraus and Kraus, 2001; Nystrom 2005).

Although urea is a perturbing solute that inhibits enzyme activity and decrease protein stability, it is employed by some species as a major osmolyte especially in the mammalian kidney cells (Singh et al. 2009; Yancey and Somero 1979, 1980a, b; Yancey et al. 1982). It gets accumulated at very high concentration ranging from 400 mM to 2 M depending on the diuretic and antidiuretic conditions in mammalian kidney cells. Concentration of urea even goes up to 3–4 M in xeric rodents under antidiuretic conditions (MacMillen and Lee, 1967). In order to circumvent the toxic effect of urea, kidney cells accumulate a second class of osmolytes called methylamines (Yancey et al. 1982). It has been observed that the optimal ratio of methylamines to urea for thermodynamic counteraction is approximately 1:2, which is the ratio generally found in tissues containing high urea levels (Yancey 2003; Yancey and Somero 1980a, b). Timasheff and co-workers have proved that at this ratio the effect of methylamines and urea are algebraically additive. This is referred to as the counteraction hypothesis.

For a number of enzymes, Yancey and Somero (1980a, b) found that urea alone generally increases K_m and decreases k_{cat}, whereas methylamine alone has the contrasting effect of decreasing K_m while increasing k_{cat}. When urea and methylamine are combined in a 2:1 urea: methylamine ratio, the effects of both solutes on K_m and k_{cat} offset one another, giving apparent k_{cat} and K_m values in the combined presence of urea and methylamine that are equal to k_{cat} and K_m determined in the complete absence of the two solutes. In terms of $\Delta G_D°$, urea decreases $\Delta G_D°$ by shifting the denaturation equilibrium, native conformation ↔ denatured conformation towards the right, and on the other hand methylamines increases $\Delta G_D°$ by shifting the equilibrium towards left. Therefore, a 2:1 ratio of [urea]:[methylamine] keeps the denaturation equilibrium unperturbed. This confirms that the occurrence of both in a single cell is a must as presence of methylamines alone makes some enzymes too rigid or too aggregated for proper functioning, while urea may restore flexibility or disaggregation of functionality.

The extent to which Yancey and Somero's counteraction hypothesis holds as a general mechanism for proteins in urea/methylamine-containing cells is still unclear (Mashino and Fridovich, 1987). The counteracting osmolyte methylamines are expected to reverse the harmful effects of urea on any protein, regardless of whether that protein evolved in the presence of the urea-methylamine mixtures. However, most studies on the counteraction phenomenon of the co-solutes on proteins have focused on enzymes from kidney or cartilaginous fishes that have indeed evolved in the presence of methylamines and urea mixtures (Burg et al. 1996; De Meis and Inesi 1988; Yancey and Somero 1978, 1980a, b). Of the small number of enzymes (that have not been involved in the urea-methylamine mixture) studied, a significant fraction of these do not exhibit counteraction (Mashino and Fridovich 1987; Yancey and Somero 1978, 1980a, b).

Studies using model protein indicate that the counteraction is not perfect but partial in nature at the ratio 1:2. There is always a lag of 1–3 °C in T_m in the presence of 2 M urea and 1 M TMAO to reach the control (Burg 1995; Lin and Timasheff 1994). Many authors also showed that the best ratio for a perfect compensation of methylamine:urea effect is 1:1 (Bagnasco et al. 1986; Baskakov et al. 1998; Qu and Bolen 2003).

In apparent contradiction to the counteracting osmolytes hypothesis, urea (1.0 M) and three different methylamines (TMAO, betaine, and GPC) have similar and inhibitory effects on aldose reductase (Burg and Peters 1997).

In a recent development, Singh et al. (2007a, b) was able to show that the urea-methylamine counteraction at 2:1 ratio is protein specific. The group identified that RNase-A and lysozyme is partially counteracted by urea:methylamine mixture at 2:1 ratio which they observed perfect counteraction in case of α-lactalbumin. They argued that the main origin of the observed difference in counteraction is due to the variation in the structural characteristics of the denatured state of the proteins.

3.8.4 Glycerol Is the Main Osmolyte to Protect from Cold and Freezing Stress

Cold and freezing stress is also one of the major challenges encounter by various organisms. Destabilisation of cell membranes and the formation of ice in intercellular spaces, and the consequent cellular dehydration, due to movement of intracellular water to the extracellular space are mainly caused by cold stress (Kosová et al. 2007; Uemura et al. 2003; Xin and Browse 2000). Solutes like glycerol, trehalose helps to cope up with such damages. Most animals including gall moth (*Epiblema scudderiana*), caterpillars (Storey and Storey, 1996), rainbow smelt, gall fly (*Eurosta solidaginis*) larvae and intertidal barnacles use glycerol against cold and freeze stress. Wood frogs (*Ranasylvatica*) use glucose and New Zealand alpine wetas (*Hemideina maori*), use trehalose (Neufeld and Leader 1998; Storey and Storey, 1996) under extreme cold. Certain amino acids such as proline also accumulate in some freeze-tolerant animals (Neufeld and Leader 1998; Storey and Storey, 1996) and several freeze tolerant plants (Hare and Cress 1997; Xin and Browse 2000). Glycine betaine is an important osmoprotectant in *B. subtilis* for existing in the cold environment (Hoffmann and Bremer, 2011).

3.8.5 Glycine Betaine Protects Macromolecules from Salt Stress

Prokaryotic microorganisms dominate other forms of life in concentrated salt solutions (salt lakes, coastal lagoons). They require to accumulate compatible solutes to cope up with the high concentration of NaCl which would otherwise leads to the disruption of macromolecule function. Glycine betaine is accumulated in *Sinorhizobium meliloti*, the root symbiont of the legume crop alfalfa when subjected to salt stress (Talibart et al. 1997). Glycine betaine is also a very good salt protectant in bacteria. Euryhaline alga *Dunaliella* use glycerol to offset the deleterious effects of KCl and NaCl (Borowitzka and Brown 1974). Many salt tolerant plants accumulate significant amounts of proline and glycine betaine in response to high salinity (Bohnert et al. 1995; Di Martino et al. 2003; Rhodes and Hanson 1993; Yancey et al. 1982). Enzyme activity measurements revealed that other methylamines including sarcosine and dimethylglycine can counteract NaCl inhibition (Pollard and Wyn Jones, 1979).

3.9 Osmolytes Can Correct Protein Misfolding Defects and Reverse Aggregation

Adverse cellular or physiological factors and many inborn errors of metabolism may compromise correct folding of nascent polypeptide chains into the biologically active, three dimensional structure of the native state. A broad range of human diseases arises from such misfolded proteins which are commonly referred as protein misfolding diseases. Such diseases mainly result from three main pathological states. One is due to impairment in the folding efficiency of a protein resulting production of proteins with reduced functional quality and therefore degraded via the quality control system or may be due to the improper trafficking of a protein. The largest group of misfolding diseases is associated with the conversion of

proteins from their soluble functional states into highly organized fibrillar aggregates (Chiti and Dobson 2006). Examples of such protein misfolding diseases include Alzheimer's disease, transmissible spongiform encephalitis, Huntington's diseases, cystic fibrosis, diabetes type II, amylotropic lateral sclerosis and Parkinson's disease (Soto 2001). Recently, it has been shown that chemical chaperones (osmolytes) are able to correct such misfolded conformations to prevent the excessive degradation of various mutant proteins and consequently promoting the intracellular functional activity of the mutant proteins (Loo and Clarke 1997; Sato et al. 1996; Tamarappoo et al. 1999). Not much is known about their direct role in the folding process but they appear to influence the rate or fidelity of the protein folding reaction. A list of diseases caused due to protein misfolding and aggregation is given in Table 3.3. It is seen in Table 3.3 that there are at least three different types of aberrant proteopathy, i.e. misfolding, aggregation and enhanced degradation. Several osmolytic compounds have been tested for their possible effect on all these different proteopathies. It has been observed that osmolytes like glycerol and TMAO are effective in general towards any type of proteopathy and protein chosen (Burrows et al. 2000; Howard and Welch 2002; Leandro et al. 2001; Powell and Zeitlin 2002; Yoshida et al. 2002). Further it can also be noted that some osmolytes like trehalose, galactose, sorbitol and proline are specific against the type of the protein misfolding and hence the type of pathogenesis (Ishii et al. 1993; Singh et al. 2007b).

Osmolytes are not only known to have altered protein misfolding but also can alter amyloidogenic processes. Therefore they are promising therapeutics for neurodegenerative diseases. Osmolytes like TMAO and proline are reported to induce nonamyloidogenic and non-toxic amorphous aggregates of mutant Huntingtin Exon 1 (Borwankar et al. 2011). Tanaka and colleagues (Tanaka et al. 2005) were able to show that various disaccharides particularly trehalose had the potential to minimize the aggregation propensity of myoglobin and increase the stability of the partially unfolded protein. Also, trehalose was effective in slowing down the aggregation of several aggregation prone proteins insulin (Arora et al. 2004) and W7FW14F (Vilasi et al. 2008). Yang et al. 1999 showed that TMAO and glycerol control fibril assembly of amyloid-β (Aβ) which is a defining characteristic of Alzheimer's disease (Yang et al. 1999). Further, TMAO protects MC65 cells under conditional expression of amyloid protein precursor carboxyterminal fragments (Woltjer et al. 2005).

Some osmolytes show concentration dependent effect on the action of protein aggregation. For instance, betaine depending on its concen-

Table 3.3 Effect of osmolytes on various human protein misfolding diseases

Osmolyte	Disease	Protein involved	References
Glycerol, TMAO	Cystic fibrosis	CFTR	Howard and Welch (2002)
Glycerol, TMAO	Nephrogenic		
	Diabetes insipidus	AQP2	Tamarappoo et al. (1999)
	Type II		
TMAO, Proline	Huntington	Huntingtin	Borwankar et al. (2011)
TMAO	Parkinson	α-synuclein	Uversky et al. (2001)
TMAO, glycerol	Alzheimer	β-amyloid	Yang et al. (1999)
TMAO, glycerol	Homocystinuria	CBS	Singh et al. (2007a, b)
Sorbitol, proline			
Glycerol	α1-antitripsin deficiency	α1-AT	Burrows et al. (2000)
Glycerol	Phenylketonuria	PAH	Leandro et al. (2001)
Galactose	Fabry	GLA	Ishii et al. (1993)
TMAO, DMSO	Machado-Joseph	Ataxin-3	Yoshida et al. (2002)

tration can either induce protein aggregation or disrupt preformed large aggregates (Natalello et al. 2008). Low concentration (5–7.5 mM) forms large insoluble aggregates of GST-GFP (glutathione S-transferase-green fluorescent protein) and high concentration (10–20 mM) formed soluble protein assemblies. Similar case is of the intrinsically disordered protein α-synuclein, where 3 M TMAO forms a relatively heterogenous ensemble of compact oligomeric structures, while at lower concentrations accelerate fibrillation (Uversky et al. 2001). Moreover, 50 mM trehalose makes Aβ42 peptide form toxic oligomers (Liu et al. 2005) while 250 mM trehalose makes fibril formation slower (Qi et al. 2009). An example of complete inhibition of protein aggregation is given by the amino acid osmolyte proline, in globular proteins such as lysozyme (Samuel et al. 2000) and P39A cellular retinoic acid-binding protein (Ignatova and Gierasch 2006). Also report is available for aggregation inhibition of Aβ40 by trehalose (Kim et al. 2010).

Further many small saccharides are also reported to show some effects on fibrillations of Aβ40 and Aβ42. Galactose and mannose induces the aggregation of Aβ40 and Aβ42 into mature fibrils while glucose, sucrose and fructose induce oligomerization (Fung et al. 2005). Using glucagon as a model system of fibrillation, Macchi et al. 2012 showed that osmolytes like ectoine, betaine, sorbitol and sarcosine accelerate its fibrillation while taurine slowers it down (Macchi et al. 2012).

3.10 Effects of Osmolytes on Nucleic Acids

Till date, most of the studies on the effects of osmolytes on macromolecular structure and function have been based solely on protein molecules. A little attention has been given on their effects on nucleic acids. Not many reports are available regarding the studies of effects of these compounds on nucleic acids. The beneficial effects of these compatible solutes on nucleic acids and nucleic acid/protein complexes have been mainly derived from improvements in yield and specificity of polymerase chain reaction (PCR). Furthermore, the effects also extend to nucleic acid structural and thermodynamic stabilization, improvement of protein/nucleic acid complex formation, nucleic acid purification and cell free transcription, as well as modulation and regulation of restriction enzyme function (Kurz 2008).

Rajendrakumar and co-workers have carefully investigated the effects of several osmolytes (proline, hydroxyproline, glycine, alanine, valine, leucine, serine, betaine, D-glucose and sarcosine) on the stability of DNA. It was found that different osmolytes have different effects on DNA stability. Proline and glycine betaine were found to have a destabilizeing effect on the double helix and hence lower the T_m of DNA in a concentration dependent manner (Rajendrakumar et al. 1997; Rees et al. 1993). Amino acid osmolytes (alanine, valine, leucine) bring about no significant changes in the stability of the DNA (in terms of T_m) even at high concentrations. However, 2.0 M hydroxyproline reduces T_m by 8.0 °C. Proline (1.0 M) reduces T_m of DNA from calf thymus considerably (by 6.0 °C). Betaine at a similar concentration also reduces the T_m of poly (dG-dC) by 5.0 °C and that of bacterial DNA by 4.0 °C (Rees et al. 1993). Interestingly, proline (1.0 M) was also observed to counter the effect of NaCl (0.5 M) and spermidine (10 mM) on DNA stability (Rajendrakumar et al. 1997). However, glycine could not counter the effect of sodium.

At physiological pH, DNA exists as a highly charged anion; hence, it is expected to be surrounded by cations which have a natural binding affinity. Salts under high salinity stress conditions should stabilize the double helix, which could adversely inhibit the DNA function in replication and transcription (Csonka 1989). Presumably, a mixture of proline (or betaine) and NaCl counterbalances each other such that the DNA stability is not affected. Furthermore, *E. coli* cells grown at saline conditions were found to actively accumulate and concentrate

glycine betaine as much as 105 times that of the growth medium (Rudulier et al. 1984). In addition, the presence of high internal concentrations of glycine betaine under the stress-adapted conditions is found to reverse the effects of salinity mediated osmotic stress on DNA replication and cell division in *E. coli*, which further supports the role of betaine in alleviating the stress effects on DNA function (Meury 1988). Thus, the selective accumulation of these two organic osmolytes (proline and glycine betaine) in a wide range of organisms under the salanity stress appears to be a conserved adaptive measure. Hence, one can speculate that if the effects of osmolytes are to enhance only the macromolecular stability, it would have not been possible for the cells to counteract the effects of salts on nucleic acids. More recently (but to a lesser details), data are available for complex formation between the leucine-responsive regulatory protein (LRP) and ribosomal DNA (rDNA). Here the osmolyte ectoine was found to stabilize the complex while the amino acid leucine had a destabilizing effect (Pul et al. 2007).

Similar to the observations on DNA, osmolytes also destabilize the structure of RNA. Recently, the effects of nine different osmolytes were investigated on the secondary and tertiary structures of several RNA structures. All these osmolytes were reported to destabilize the secondary structure of RNA, although to different extents (Lambert and Draper 2007). It was observed that osmolytes may have either stabilizing or destabilizing effects on the tertiary structures of RNA. Urea and proline are found to be uniformly destabilizing in their effects. Interestingly, there exist considerable variations among RNAs in the effectiveness of a particular osmolyte. For example, the U1061A and GACG variants of the rRNA fragments, which differ by only four nucleotides in sequence, differ by nearly a factor of two in their sensitivity towards methanol and ethylene glycol (Lambert and Draper 2007). This differential effect of a particular osmolyte on the secondary and tertiary structures of RNA was explained on the basis of preferential hydration effect. As these osmolytes mainly favour burial of the RNA backbone, i.e., favourable osmolyte interactions with base surfaces must be outweighed by unfavourable interactions with backbone.

3.11 Pharmacological Application of Osmolytes

One of the major challenges faced by pharmaceutical companies today is protein instability and loss of activity upon transportation to long distances. Osmolytes are attractive molecules that have proven useful to maintain protein stability by increasing T_m to protect from various denaturing conditions. Many therapeutically important proteins available in the clinic are extremely costly as the proteins have to be extracted from the blood or tissues. This can in fact be replaced by recombinant production of these proteins to bacteria. Major hurdle faced by pharmaceutical companies for the overproduction is that most of these proteins go to inclusion bodies upon over expression in bacteria. It has been demonstrated that some osmolyte mixtures or individual osmolytes are of great success to remove inclusion bodies and enhance soluble protein yield (Tsumoto et al. 2004). On the other hand, osmolytes like betaine, glycine, sarcosine and proline have proved to be effective in enhancing monoclonal antibody production by hybridoma cells (Oyaas et al. 1994). One great success that has been observed in health industry is that many osmolytes can remove or inhibit aggregation or amyloid formation in vitro and therefore use of osmolytes will be a good strategy to fix human genetic disorders including neurodegenerative and metabolic disorders. However, their use in in vivo condition has been challenged as this chaperones work in high concentration which might be too toxic for the cells (Arakawa et al. 2006). In addition to the protein conformational diseases, some osmolytes are also clinically important for various numbers of diseases. For example, sorbitol plays a key role in protecting renal failure and effect of high dosage of NSAIDS in kidney (Moeckel et al. 2003).

3.12 Conclusion and Future Perspectives

There have been several significant landmark findings, and several questions have now been addressed due to the advancements in understanding the effects of organic osmolytes for their role in protein folding/stabilization and their protecting mechanisms of cellular macromolecules under stress conditions. One general conclusion can be drawn, i.e., organic osmolytes are accumulated in response to stress and they provide a generic stabilization to the cellular macromolecules. Advances in the recent molecular dynamic simulations and computational analysis techniques have magnified our understanding of mechanisms of protein-osmolytes interactions and osmolytes-induced protein folding/stabilization at molecular levels. Keeping aside the generic role of osmolytes in stabilization of cellular macromolecules, recent advances in the knowledge of osmolytes and their possible use in pharmaceuticals seem quite promising. Individual osmolytes (or mixtures) have now been shown to posses the ability of removing inclusion bodies and enhance soluble protein yield (Tsumoto et al. 2004). In addition to this, a number of organic osmolytes have also been shown to be effective in correcting protein misfolding and also in altering amyloidogenic progressing of misfolded protein which would thereby lead to a number of proteopathies. Thus, they can be promising candidates for therapeutic interventions of various human genetic defects and neurodegenerative diseases. These informations can be utilised aptly by researchers working in pharmaceutical and clinical industries for their potential applications for the use of these osmolytes as therapeutic for human diseases. In the near future, these so called "chemical chaperones" might change the mode of treatment of several human diseases and provide new aspects of research in biotechnological, pharmaceutical and clinical fields.

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Part II

Proteopathy: Failure of Proteostasis

Protein Folding and Aggregation: A Revisit of Basic Conception

4

Atiyatul Qadeer, Nida Zaidi, and Rizwan H. Khan

Contents

Abstract

In this chapter, an overview of protein folding and aggregation is given. Starting from the earliest concept of sequential or hierarchical pathways to the current view of folding funnel hypothesis, several different models that have been proposed in connection with protein folding phenomena are discussed. An account of 'molten globule' is given with respect to its structure, characterization, role in protein folding and possible physiological significance. Protein aggregation in relation to importance of such studies in medicine and biotechnology, various classes of protein aggregates, their structure and morphology, proposed mechanisms of aggregation along with an account of techniques that are used to characterize and aggregates are also discussed. We have also summarized various intrinsic and extrinsic factors that are known to influence aggregation of proteins alongside different strategies that have been proposed to inhibit the self-assembly process of protein aggregation.

Keywords

Molten globule • Protein aggregation • Protein folding • Amyloid formation

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4.1 Introduction

The folding of a protein into its native functional conformation is a challenging task *in vivo* and *in vitro*; the failure to do so may result in protein misfolding or aggregation. The aggregates formed may be organized into fibrillar structures known as 'amyloid fibrils', which can be detected by spectroscopic and microscopic techniques. The misfolding and subsequent aggregation of proteins is an important area of intensive research as it is directly related to several pathological conditions and a great hurdle in production of therapeutics in biopharmaceutical industry. Thus, a current understanding of fibril formation mechanisms and finding methods for intervention are topics of utmost investigation. However, whatever prevention/moderate inhibition has been obtained so far is mostly experimental. Therefore in the current scenario, an understanding of basic concepts starting from the earliest concept of hierarchical pathway of protein folding to the forces involved, characteristics of molten globule state and current perspective of protein misfolding and aggregation is crucially required for therapeutics development against devastating diseases.

4.1.1 Protein Folding

Proteins are the crucial component of biological machinery that runs living organisms. These biomolecules, having diverse structure and functions, are synthesized in cellular factories known as ribosomes. The linear covalent structure of polypeptide produced by the ribosomes is ultimately headed towards specific native conformation. This process is referred to as 'protein folding' and is directed by the sequence of amino acids in a polypeptide. To understand the mechanism of protein folding is one of the greatest challenges. Generally, every protein has its unique amino acid sequence that folds to give specific three-dimensional structure which determines the functional diversity among protein molecules.

4.1.1.1 Forces Underlying Protein Folding and Structure

The core of a globular protein comprises mainly of hydrophobic amino acid residues while their surface is occupied by polar residues. The peptide backbone as well as side chains which are considerably mobile in an unfolded polypeptide become somewhat fixed as a result of folding. This is due to the interplay of specific intramolecular forces and energies, as shall be discussed briefly.

Hydrophobic Effect

The energetic consequences of removing apolar group from the interior of a protein to its surface is broadly referred to as the hydrophobic effect. Earlier, hydrophobic effect used to be considered mainly an entropic effect arising from restructuring of water molecules around the apolar surface. As this hydration process is energetically unfavourable, it causes the polypeptide to undergo a conformational change so as to minimize the exposed surface area. Later studies have revealed that hydrophobic effect is actually an outcome of both hydration (entropic effect) as well as interaction between solute molecules via van der Waals forces (Makhatadze and Privalov 1995). The strength of hydrophobic effect depends upon the temperature such that at low temperature entropic contribution exceeds while at higher temperature enthalpic contribution is predominant (Schellman 1997). The hydrophobic effect is considered as the major driving force of protein folding, as it results in rapid collapse of the polypeptide chain and thus increases the rate of folding by reducing the configurational space (Dill 1990).

Hydrogen Bonding

Hydrogen bonds are non-covalent bonds that involve partial sharing of hydrogen atoms between a donor such as hydroxyl (–OH) or amine (–NH) group and an electronegative acceptor atom, mainly oxygen or nitrogen. These constituents are abundantly present in protein molecules such as peptide backbone (–NH as hydrogen donor and –C=O as hydrogen acceptor)

and side chains of several amino acid residues. Although perceived as charge-charge interaction, hydrogen bond is actually a dipole-dipole interaction involving permanent dipoles.

Electrostatic Interactions

Electrostatic interactions require their partners to exist in ionized form such as NH_3^+ and COO^- groups of main peptide along with the side chains of polar residues (Azia and Levy 2009; Marshall et al. 2002). These interactions are largely specific in nature unlike non-specific hydrophobic forces and hydrogen bonds and thus play leading roles in protein conformation and function determination. Depending upon their ionization states, electrostatic interactions may either stabilize or destabilize protein conformation. For instance, salt bridges have been found to contribute significantly to the thermal stability of thermophilic organisms (Lam et al. 2011).

Configurational Entropy

Native structures of proteins are stabilized by the intramolecular interactions whereas destabilized by configurational entropy. It is due to the fact that on folding of unfolded polypeptide into native state configurational entropy or degree of freedom available to the protein decreases. This decrease comes from restricted mobility of both polypeptide backbones as well as side chains (Jaenicke 2000). It is obvious that peptide backbones of amino acid residues buried within the core of the protein will experience lesser degree of freedom than those located on the surface. Additionally, change in the configurational entropy is more pronounced for the burial of a side chain as these have substantial flexibility on the surface. The entropic change is also determined by amino acid composition. For example, a proline-rich polypeptide in unfolded state will have lower entropy and thus be more stable in folded state. However, the concept gets reversed for glycine-rich polypeptide.

4.1.1.2 Protein Folding Dilemma

Anfinsen's famous experiment (Anfinsen 1973), a plausible extension of the central dogma, would be the addition of the concept that amino acid sequence of protein encodes its three-dimensional structure. This remarkable observation has now recommended for considering the 'second half of the genetic code'. To put it simply, protein folding problem refers to the query as to how the information contained within the amino acid sequence is translated into the three-dimensional structure.

If one assumes that polypeptide chains randomly search for all the possible configurations they can acquire and if each peptide bond has three possible states, than total number of the configurations searched by a small polypeptide of 100 amino acid residues will be $3^{100} = 5 \times 10^{47}$. The time taken to try all these configurations at the rate of 10^{13} per second would be 10^{27} years. This contradiction was reported by Cyrus Levinthal in 1969 and is now called as 'Levinthal's paradox' (Levinthal 1969). It is now clear that protein folding cannot proceed via random sampling of all the conformations. The specific folding pathway existence idea inspired experimental investigations of specific folding intermediates and led the proposal of several models of protein folding.

4.1.1.3 Models of Protein Folding

Diffusion-Collision Model

Proposed by Karplus and Weaver (Karplus and Weaver 1994), diffusion collision (DC) model assumes proteins to be divided into different parts such that amino acid sequence of each part contains information sufficient to fold independently. These parts which are called 'microdomains' may be portions of incipient secondary structures or hydrophobic clusters (Fig. 4.1a). The microdomains move diffusively and undergo microdomain-microdomain collision such that productive collision leads to coalescence of the microdomain into larger units. These multimicrodomain intermediates then undergo mutual rearrangement to form the native tertiary structure. The diffusion process is assumed to be the rate-limiting step. Two main inferences can be drawn from DC model: (1) protein folds through series of intermediate states and (2) secondary structure elements are formed before tertiary structure. DC model has been supported by several experimental studies, for example, apomyoglobin (Nishimura et al. 2000), barnase (Wong et al. 2000) and engrailed homeodomain (En-HD) (Mayor et al. 2003) etc.

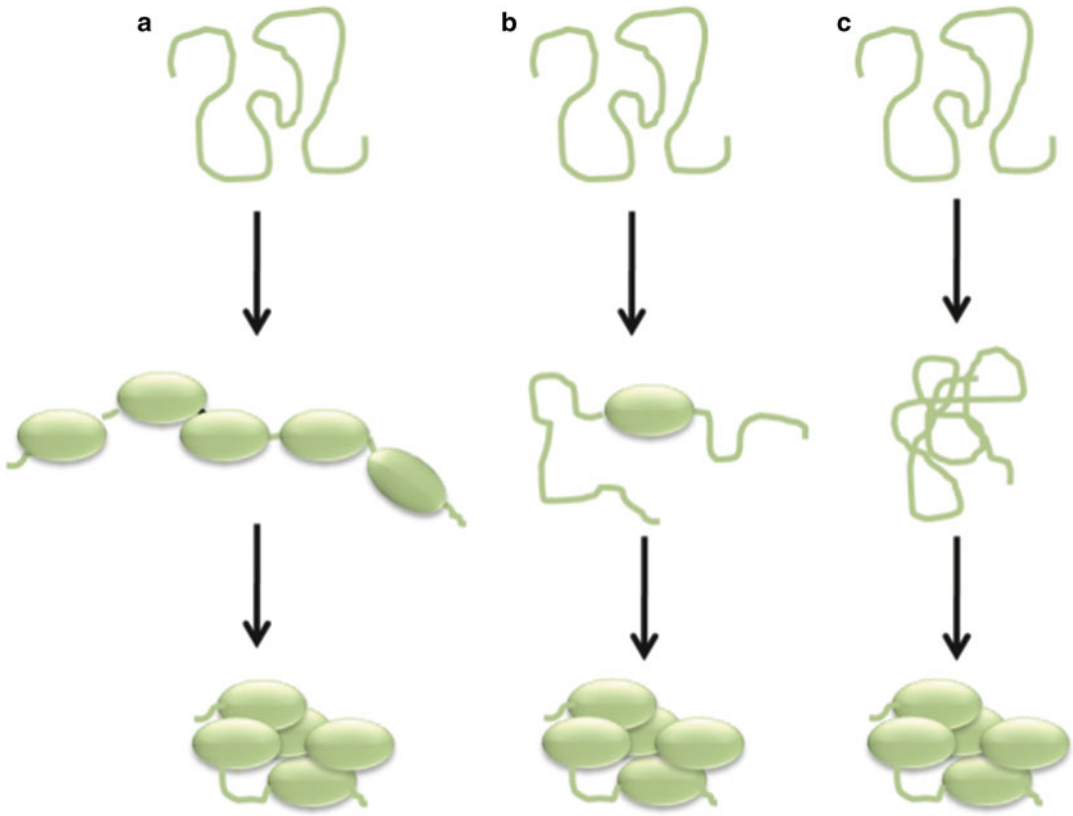

Fig. 4.1 Protein folding models. (**a**) Diffusion-collision model (**b**) Nucleation-condensation model and (**c**) Hydrophobic collapse models

Framework Model

This model was given by Baldwin (Baldwin 1989): protein folding is a process in which formation of native secondary structure precedes formation of tertiary contacts. In principle, the framework model is similar to the DC model in the sense that both assume protein folding to proceed in stepwise manner where local structures are formed initially and their subsequent interaction or collision leads to the formation of more complex structure.

Nucleation-Condensation Model

The nucleation-condensation (NC) model of protein folding was given by Fresht (1995). NC model states that folding begins with a formation of marginally stable nucleus that involves small number of amino acid residues, about which the rest of the molecule condenses rapidly (Fig. 4.1b). Thus in the folding reaction, number of required conformations to be sampled gets significantly reduced. NC model holds a key feature that both secondary and tertiary contacts are formed simultaneously to form the nucleus. This contrasts with DC model which assumes folding to be a hierarchal procedure where secondary structure is formed independent of the tertiary structure, Thus, the two models differ on whether protein folding is a sequential (DC model) or a concerted (NC model) process.

Jigsaw Puzzle Model

It was proposed by Harrison and Durbin in 1985 (Harrison and Durbin 1985). This model opposes the existence of single definitive folding pathway involving sequence of events and states that protein folds by large number of different, parallel folding routes just like multiple ways to solve a jigsaw puzzle. This model also helps to understand why mutations do not adversely affect native structure of proteins. This model is actually consistent with the latest 'energy landscape' view of protein folding as shall be discussed later.

Hydrophobic Collapse Model

This model is based on the idea that, since the interior core of the native state of protein contains mainly the hydrophobic residues while charged residues are on the surface, therefore an initial event of the folding of a nascent polypeptide chain must be rapid hydrophobic collapse that results in the formation of a collapsed intermediate (Dill 1985). Subsequently, the secondary or the tertiary structures are formed within this intermediate (Fig. 4.1c). This model highlights the 'earliest event' of the folding, as the side chains of hydrophobic residues are pulled away from water in the same way as the oil droplets get clumped together in water.

It should be noted that the above described folding models are not mutually special but rather are interconnected in some or the other way. Each model attempts to highlight different aspects of protein folding, and also, experimental evidences have been provided in support of each of them.

Folding Funnel Hypothesis and Free Energy Landscape

It is clear that protein folding is a complicated process involving series of events. Attempts have been made to solve this complexity by applying statistical approach (energy landscape) to the energetics of protein conformation (Bryngelson et al. 1995; Frauenfelder et al. 1991).

Energy landscapes are the mathematical tools that provide an insight into the microscopic behaviour of a molecular system. Energy landscape of protein-solvent system is defined by an energy function, $F(x)=F(x_1, x_2, \ldots, x_n)$, where $x_1,\ldots, x_n$ are the variables describing microscopic state of the protein and 'n' is the degrees of freedom. $F(x)$ is usually defined as the free energy of a given conformation of the protein, where the entropic part is contributed from all possible configurations and conformational states of solute and solvents and the enthalpic part contributed from the non-covalent bond formation and loss within the protein and between protein and solvent.

For polypeptides with random amino acid sequences and vague stable conformation, the energy landscape may either be very rugged containing too many local minima or very large flat energy surface (Plotkin et al. 1996). Real proteins, on the other hand, are not random as they contain optimized sequences, acquired during the course of evolution, and thus efficiently and rapidly fold into well-defined stable conformation (Onuchic and Wolynes 2004). Tilt and funnel-shaped energy landscape depicts the ability of proteins to fold quickly and efficiently.

Furthermore, a funnel-shaped energy landscape means that protein structure's free energy is inversely proportional to its closeness to the native state as shown in Fig. 4.2. It also implies that protein folding can proceed via multiple routes (as also postulated by jigsaw puzzle model). The broad top of the funnel represents a large ensemble of unfolded polypeptides with high free energy and conformation entropy. As folding proceeds, there occurs a progressive reduction in the number of accessible conformational states such that at the narrow bottom, there remains unique native state having minimum free energy as well as conformational entropy. This makes the shape of the protein folding landscape look like a funnel.

The most realistic protein folding funnel is the one which carries information about kinetics, barrier heights, smoothness and shape of the landscape. The funnel will become shallow under inappropriate folding conditions, causing the polypeptide to remain around the upper part of the funnel leading most of the time to slow folding (Dill and Chan 1997). Once the appropriate conditions are provided, funnel gets stretched down forming a steep slope and consequently favors the molecule's downhill movement towards native state.

Different scenarios of the folding represented by different folding models help in understanding protein folding. The funnel-shaped energy landscapes further help in characterizing several events such as molten globules and transition state ensemble formation, folding intermediates trapping in the local minima, tertiary interaction reconfiguration and various thermodynamic as well as kinetic aspects of protein structure. Therefore, this latest view has subsided the classical folding pathway model.

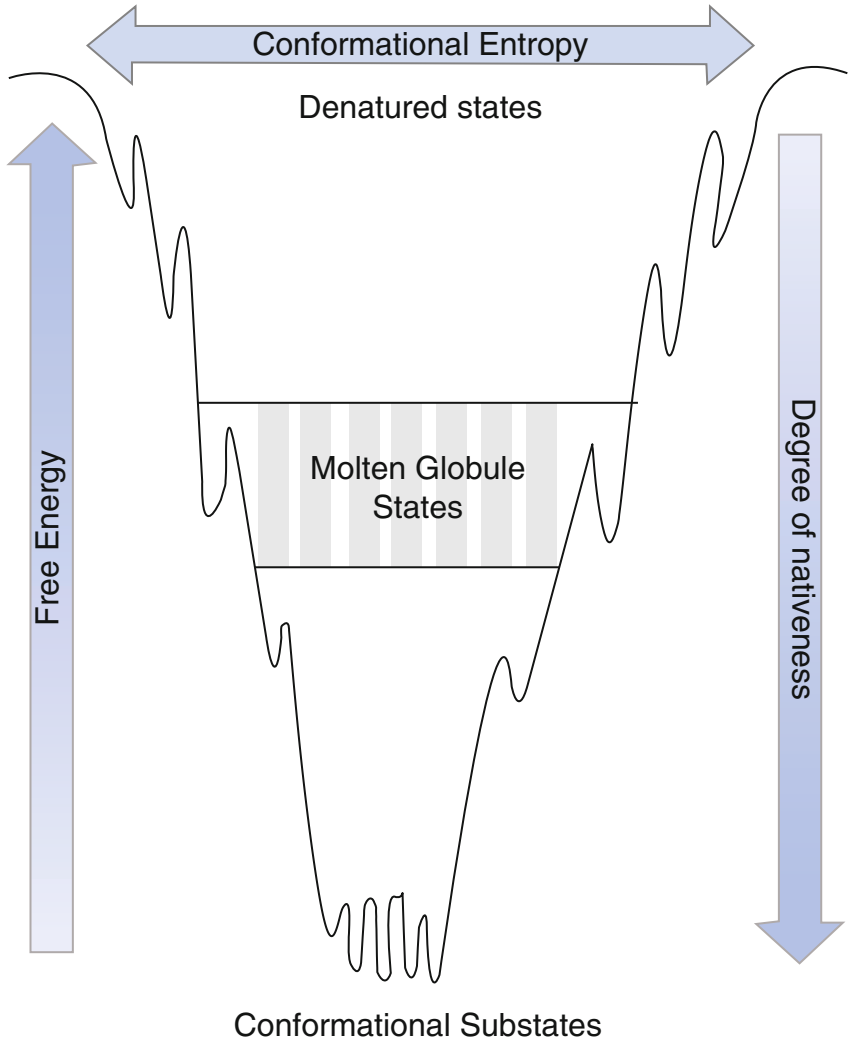

Fig. 4.2 Funnel-shaped free energy landscape model unveiling the detailed processes of the folding reaction

4.1.1.4 Molten Globules

As discussed earlier, 'Lavinthal's paradox' (1969) inspired folding studies on a number of globular proteins to detect the specific 'folding intermediates' (Kuwajima et al. 1976; Nozaka et al. 1978; Wong and Tanford 1973). Several equilibrium intermediates were traced out from different proteins having considerable structural similarity. In 1983, Oghushi and Wada proposed the term 'molten globule' for the equilibrium intermediates and related them to the general physical state of globular protein (Ohgushi and Wada 1983).

Molten Globule (MG) State Characteristics

According to Kuwajima (Arai and Kuwajima 2000), the common features of MG state are-

- Presence of substantial amount of secondary structure that can be observed by far-UV circular dichroism (CD) and Fourier transform infra-red (FTIR) spectroscopy.
- Absence of most of the specific tertiary contacts and loss of tight packing of the side chain residues as evidenced by Trp fluorescence or near-UV CD or NMR.

- Compactness closer to the native state with 10–30 % larger radii. Intrinsic viscosity, dynamic light scattering (DLS), size exclusion chromatography (SEC) and small-angle X-ray scattering can be utilized to monitor compactness.
- Presence of loosely packed solvent-accessible hydrophobic core. It can be detected by hydrophobic dyes.

Hydrophobic dyes include ANS which is frequently used to determine the presence of MG state, owing to their preferential binding to the exposed hydrophobic patches on intermediate as compared to the native or denatured state. However, ANS fluorescence alone is not sufficient to characterize folding intermediate as molten globule in the absence of other criteria. This is due to the fact that under conditions such as low pH, ANS can also interact electrostatically to the positively charged centers of protein (Arg, Lys, His) through its sulfonate (SO_3^-) moiety, thereby increasing the fluorescence intensity (Kirk et al. 1996; Matulis and Lovrien 1998). Besides, ANS tends to increase the propensity of molten globule and other compact denatured states to aggregate which may also be responsible for the observed enhancement in fluorescence intensity (McDuff et al. 2004; Povarova et al. 2010).

Models of Molten Globule

Traditionally, it was assumed that in MG conformation, backbone of protein has a native-like fold while side chains remain in a disordered state (Ptitsyn 1992) as shown in Fig. 4.3b. This view was challenged by energy landscape theory, and it was argued that how come backbones remain ordered when the side chains remain unordered (Arai and Kuwajima 2000; Privalov 1996). Therefore MG state must have some residual structure as shown in Fig. 4.3c.

Experimental Conditions to Generate Equilibrium Molten Globules

Molten globules and other intermediate states have now been identified for a variety of proteins. Generally, under certain conditions most of the proteins, if not all, can form such intermediate species. MG can be populated under mild experimental denaturing conditions such as high temperature/pressure/pH, moderate concentration of denaturants such as urea, guanidine hydrochloride, SDS and in the presence of various alcohols and salts etc. (Honda et al. 2014; Stojanovski et al. 2014; Haq et al. 2002; Sen et al. 2008). Most commonly, MGs have been observed under acidic conditions of pH < 3.0 and moderate ionic strength. Depending upon their behaviour under acid denaturation conditions, Fink et al. have classified proteins into three types (Fink et al. 1994):

- Type I: HCl titration of salt-free solution of protein leads to substantial unfolding at pH 2.0. Further titration with acid leads to collapse of the unfolded protein into compact intermediate or MG state, the A state at pH < 2.0, for example, cytochrome *c*, myoglobin, papain.
- Type II: Upon titration with HCl, these proteins do not unfold fully but transform directly into MG state at around pH 3.0, for example carbonic anhydrase, α-lactalbumin.
- Type III: Acid titration of these proteins does not cause their significant unfolding and they remain native-like to pH as low as 1.0, but in presence of urea such proteins may behave similar to type I, for example ubiquitine, concanavalinA, β-lactoglobulin.

Role of Molten Globule in Protein Folding

The MG state is assumed to be identical to the transient intermediates formed during protein folding. Using stopped-flow CD technique, Kuwajima and co-workers demonstrated the formation of an intermediate during refolding of α-lactalbumin, whose spectral properties resembled that of its A state (Kuwajima et al. 1985). These and other carefully done kinetic studies on the refolding of proteins such as interleukin-1β, staphylococcal nuclease and apomyoglobin (Heidary et al. 1997; Jamin and Baldwin 1998;

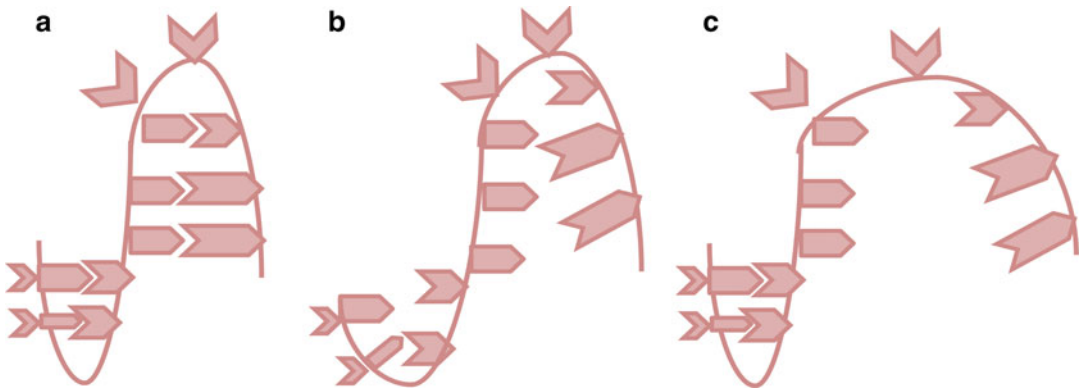

Fig. 4.3 Illustration of different models for the molten globule structure. Backbone of the protein and the side chains represented by thick line and hanging pieces of various shapes respectively (**a**) Buried side chains fitting closely together in native state (**b**) molten globule model: backbone fold is native-like but the side chains are only loosely packed (**c**) One-half of the molecule is fully folded, while the other half is completely unfolded

Maki et al. 1999) yielded firm evidences in support of the notion that in most of the cases MG state is the on-pathway productive folding intermediate.

Possible Physiological Relevance of Molten Globule

Experimental evidences suggest that molten globule conformations are involved in membrane translocation. Further, the A-state of α-lactalbumin is found to induce fusion of the phospholipid vesicles. The MG conformation of diphtheria toxin (protein), formed in the vicinity of pH 5.0, is correlated with its fusion with membranes (such as those of lysosome where low pH condition predominates) (Dumont and Richards 1988). Similarly, membrane insertion of colicin A has been related with its transition into molten globule (Muga et al. 1993). Additionally, molten globule-like conformations of newly synthesized polypeptides are expected to bind to the molecular chaperones (Fink 1995).

4.1.2 Failure to Undergo Correct Protein Folding Leads to Protein Misfolding and Aggregation

Since the correct folding of protein into its native functional conformation is a challenging task inside the crowded milieu of the living cell, the failure to do so may result in 'protein misfolding diseases'. There are several misfolding diseases with different mechanisms including Alzheimer's, Parkinson's, Huntington's, Creutzfeldt-Jakob disease, type II diabetes, etc. to name a few (Swart et al. 2014). The majority of these are associated with the aggregation of proteins into organized fibrillar structures known as 'amyloid fibrils' which get deposited at different locations inside the body. Such an unwanted aggregation may be a consequence of (1) loss of cellular protein quality control, (2) inability of ubiquitine-proteosome complex to degrade and eliminate misfolded aggregation prone molecules, (3) inefficient functioning of molecular chaperone machinery, (4) normal cellular transport of protein being hampered, (5) inappropriate protease activity producing amyloidogenic fragments of protein, (6) destabilizing mutations and so on (Balch et al. 2008). The accumulation of aggregates may take place both extracellularly and intracellularly based on ability to be stained by iodine structured mass in human tissue termed as 'amyloid' by Rudolf Virchow (Falk and Skinner 2000). The proteinaceous nature of amyloid structure was proved later through direct chemical analysis.

Earlier, the ability to form well-ordered fibrillar structure attributed only the proteins responsible for pathological conditions. It has now been widely accepted that amyloid fibrils may also be

produced from proteins not related to any disease under suitable (usually non-physiological) *in vitro* conditions, implying fibrillogenesis to be an inherent property of polypeptide chain that relies upon interaction between backbone atoms rather than those of side chains (Dobson 2003).

4.1.2.1 Protein Aggregation *In Vitro*

The misfolding and aggregation of proteins does not only have pathological consequences but is also a serious problem encountered during the *in vitro* study of proteins. Aggregation of proteins at higher concentration is a huge barrier in the refolding studies (Gupta et al. 1998; Raman et al. 1996). Thermal denaturation of proteins often results in aggregation which creates hindrance in determination of thermodynamic parameters that defines stability of protein (Eronina et al. 2014; Caves et al. 2013). The purification of recombinant proteins is hampered by their aggregation in the form of inclusion bodies (Fink 1998). Moreover, protein folding is also a complication during industrial manufacturing of therapeutic proteins. During purification and processing, proteins encounter several stresses such as high temperature, high protein concentration, surface adsorption, shear strain, fermentation, freeze-thawing, drying, etc. which is likely to alter their structure making them prone to aggregation (Liu et al. 2013; Mahler et al. 2009). Besides, irreversible aggregation of proteins is also a major problem for maintaining their stability during long-term storage, shipping and handling. In these contexts, aggregation may simply refer to physical association of protein molecules with no changes in the primary structure (physical aggregation) or the chemical cross-linking of the molecules through formation of covalent bonds (chemical aggregation). Depending upon its nature as well as environmental conditions, a protein can simultaneously undergo both types of aggregation processes resulting in the formation of either soluble or insoluble aggregates. For example, by physical methods, insulin leads to formation of soluble hexamers or insoluble fibrils and chemically resulted in soluble dimmers or insoluble disulfide-bonded aggregates (Hsieh et al. 2014; Darrington and Anderson 1995).

4.1.2.2 Classification

Due to lack of precise definition, protein aggregates have been classified differently by different authors. Two broad categories of protein aggregates include *in vivo* versus *in vitro*, and fibrillar versus amorphous. For example, amyloid fibrils are fibrillar or ordered aggregates that are observed both *in vivo* and *in vitro* whereas inclusion bodies are the amorphous or disordered aggregates that are formed *in vivo*. Similarly, *in vitro* aggregates formed during refolding at high protein concentration are the disordered aggregates (Fink 1998). Other classifications have also been proposed such as physical (or non-covalent) versus chemical (or covalent) aggregates, reversible versus irreversible aggregates, soluble oligomers (dimer to 10-mer) versus insoluble particles, etc. (Cromwell et al. 2006).

4.1.2.3 Structure and Morphology

The morphology of protein aggregates, as demonstrated by experimental evidences, seems to depend upon the solution condition rather than the amino acid sequence of proteins. This is because a single protein can form both fibrillar as well as amorphous types of aggregates based upon the environmental conditions. The pH has been found to play a key role in determining the aggregate morphology as it affects the charge distribution and also degree of structural perturbation of protein. A study on seven widely different proteins by Krebs et al. has revealed that a pH which provides high net charge to the protein favours formation of fibrillar aggregates whereas protein is likely to yield amorphous aggregates if the net charge is low (Krebs et al. 2007). Similar findings have been reported in later studies (Krebs et al. 2009).

The molecular level description of the structure of aggregates particularly the pathogenic amyloid fibrils is difficult to understand due to their large size, poor solubility and non-crystalline structure. Much of the advancement in the knowledge of fibril structure is facilitated by electron microscopy, X-ray diffraction and solid-state NMR. The amyloid fibrils are characterized by well-ordered, elongated and relatively straight fibrillar structure. Electron microscopy (EM) and

atomic force microscopy (AFM) are exploited to reveal that fibrils are composed of substructure called 'protofilament'. A single protofilament is either straight/curved with a diameter of 2–5 nm. About 2–6 such protofilaments join by either twisting together like a rope or arrange laterally in a form of ribbon to construct a fibril with a diameter of 7–15 nm. The length of the fibrils is indeterminate (Serpell et al. 2000). Furthermore, X-ray diffraction studies reveal the presence of common 'cross–β' structure in which β-sheets are parallel while β-strands in individual sheet are arranged perpendicular to the axis to fibrils (Sunde et al. 1997). The distance between β-strands is 4.8 Å while separation between β-sheets is ~10 Å. It should be noted that the morphology of the fibrils generated *in vitro* also varies with solution conditions including protein concentration, pH, temperature, composition of buffer and presence of additives, etc.

4.1.2.4 Mechanism of Protein Aggregation

Philo and co-workers have described the mechanisms of protein aggregation in a very concise and illustrative manner (Philo and Arakawa 2009). These authors have proposed five possible mechanisms of protein aggregation as discussed below:

Reversible Association of Native Monomers

This mechanism of protein aggregation is demonstrated mainly by proteins which, in their native state, exhibit the tendency to undergo reversible association. This association is facilitated by presence of self-complimentary patches on the surface of the molecules such that the self-assembly of protein monomers results in formation of small oligomers (Fig. 4.4). As protein concentration increases, bigger oligomers are formed which over time become irreversible in nature. This type of mechanism is illustrated by therapeutic proteins such as insulin.

Aggregation of Conformationally Altered Monomers

Generally native conformation of protein has lesser propensity to self-associate and commencement of aggregation requires some conformational alterations such as partial unfolding of the protein molecule (Fig. 4.5). Such conformational changes are triggered by stresses such as heat, shear, pressure, change in pH, etc. This mechanism of protein aggregation is frequently observed for several different proteins such as lysozyme, cytochrome C, etc.

In case of natively unfolded proteins, the onset of aggregation requires partial folding of the molecule into a partially structured conformer (Uversky and Fink 2004, 2005).

Aggregation Through Chemical Modification

This mechanism of aggregation also commences through change in protein conformation except that the change is chemical rather than physical (as in mechanism 2) as shown in Fig. 4.6. The chemically modifying reactions include oxidation, deamidation, hydrolysis, etc. as shall be discussed later.

Nucleation-Dependent Aggregation

This mechanism of protein self-assembly is characterized by an initial lag phase which is rate-limiting and slow step of the aggregation reaction. During lag phase, the protein monomers assemble to form a nucleus as shown in Fig. 4.7. Once the nucleus of sufficient size is formed, a rapid elongation phase follows in which addition of monomers onto this critical nucleus leads to the formation of larger aggregated species or fibrils, for example, Aβ peptide, insulin, etc. In isodesmic or nucleation-independent aggregation, there is no lag phase (Kumar and Udgaonkar 2010).

Surface-Induced Aggregation

Here, aggregation of protein is triggered by interaction of monomers with surface. As a result of this reversible binding, the protein monomers

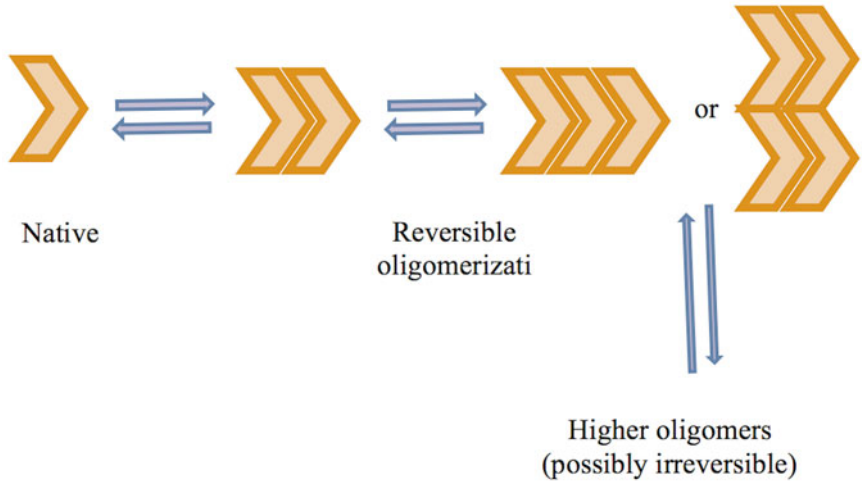

Fig. 4.4 Schematic illustration of protein aggregation through reversible association of monomers

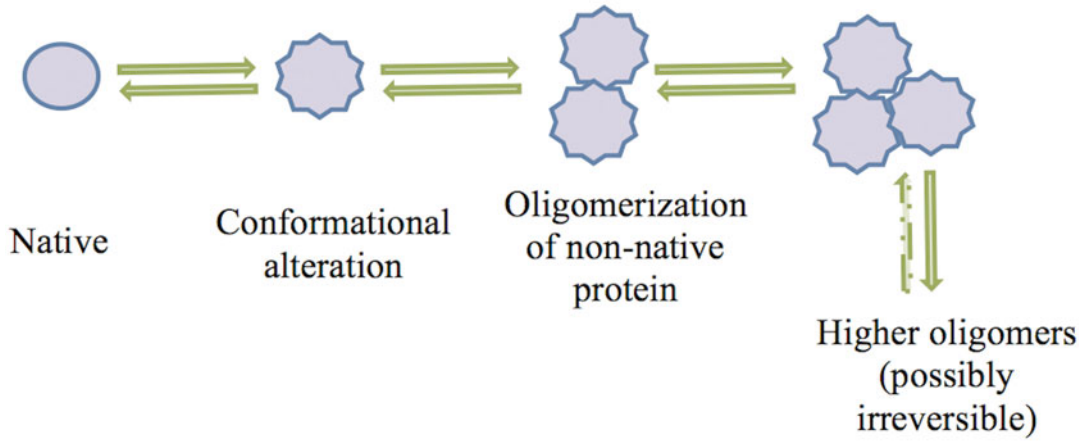

Fig. 4.5 Schematic illustration of aggregation of conformationally altered protein

undergo conformational alteration which makes them prone to aggregation either on the surface or following their release into the solution (Fig. 4.8).

4.1.2.5 Analytical Techniques for Characterization of Protein Aggregates

The characterization of protein aggregates by using combination of analytical methods is essential not only for understanding the mechanism of aggregation and designing potential drugs to combat amyloid-associated pathogenesis but also for evaluating the protein structure, biological integrity, process and product-related impurities during manufacturing and storage of biopharmaceuticals as proteins are widely used in therapeutics. The methods commonly employed in the characterization of protein aggregates including amyloid fibrils are listed in Table 4.1. The underlying principles, detailed methodology and pros and cons of all these methods have been extensively reviewed by several authors (Li et al. 2009; Mahler et al. 2009;

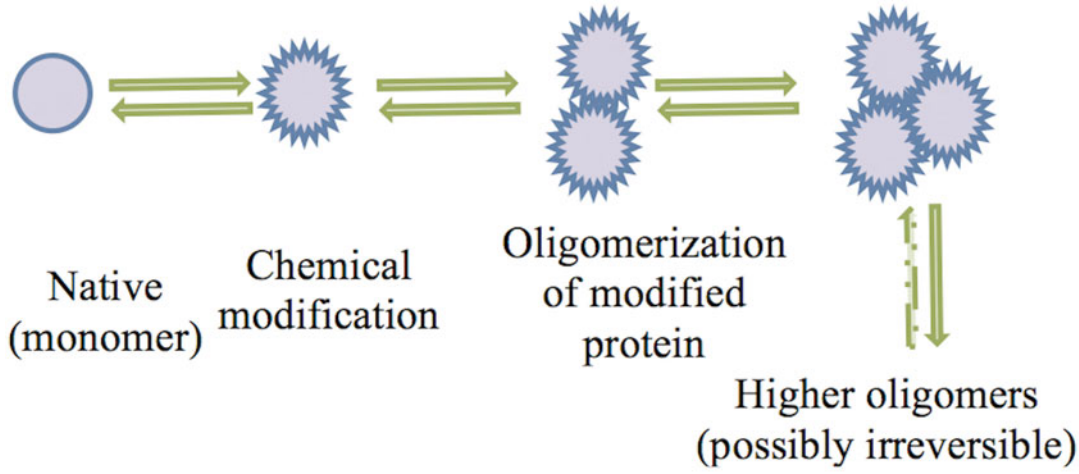

Fig. 4.6 Schematic illustration of aggregation of chemically-modified proteins

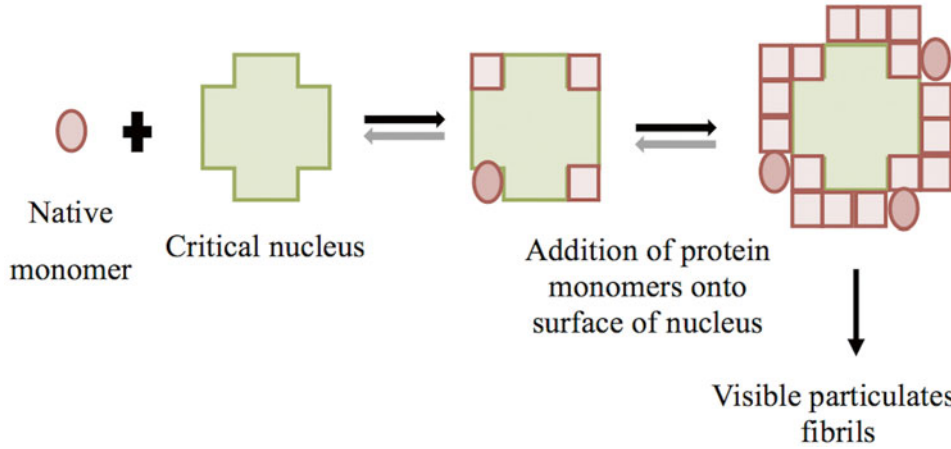

Fig. 4.7 Schematic illustration of protein aggregation through nucleation-dependent pathway

Nilsson 2004). Discussed below are the salient features of some of them.

Light Scattering

The optical properties of a solution are due to the ability of molecules or particles present therein to absorb and scatter light. Rayleigh's theory of light scattering states that particles with diameter lesser than the wavelength of incident light are able to scatter light. The upper limit on the size of particles is about 1/10 of the wavelength which implies that scattering does not take into account the shape of the particle. Light scattering is found to be an essentially employed tool in the investigation of protein aggregation. Light scattering can also be monitored through fluorescence spectrophotometer by setting identical excitation and emission wavelength, usually at 350 nm. This method has been considerably employed to detect the formation of aggregates.

Turbidity Monitoring

Aggregation of proteins into particles of various sizes gives turbid or hazy appearance to the pro-

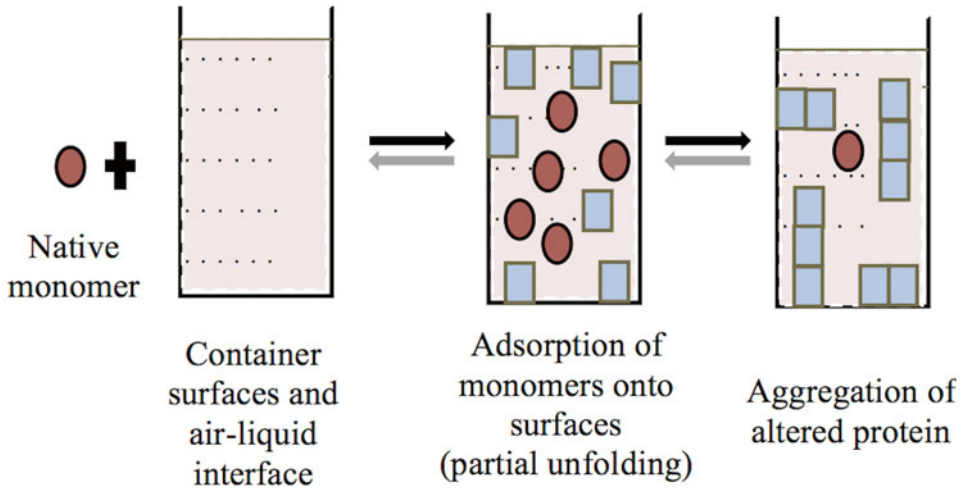

Fig. 4.8 Schematic illustration of aggregation of protein on container surfaces and air-liquid interface

Table 4.1 Analytical methods commonly used for studying protein structure, folding and self- assembly

	Monomer	Oligomers	Protofibirls/Fibrillar aggregates	Amorphous aggregates
Atomic structure	Nuclear magnetic resonance spectroscopy			
	X-ray crystallography/ diffraction			
Secondary structure	Circular dichroism spectroscopy			
	Fourier transform infrared spectroscopy			
	X-ray fiber diffraction			
	ThT fluorescence/Congo red birefringence/ Congo red binding			
	Bis-ANS/ANS/Nile red fluorescence			
Morphology	Transmission electron microscopy			
	Scanning transmission electron microscopy			
	Atomic force microscopy			
Assembly size/Size distribution	Rayleigh light scattering/turbidity measurement			
	Dynamic light scattering			
	Size exclusion chromatography			
	Analytical ultracentrifugation			
	Gel electrophoresis			

tein solution. The aggregates formed during long-term storage or other sheer stress conditions have been reported to give marked increase in turbidity (Arvinte et al. 2004; Yu et al. 2006). The turbidity can be conveniently monitored spectrophotometrically as optical density in 340–360 nm wavelength range. The time-dependant change in turbidity has also been employed as a means to study the formation or inhibition of aggregates under various conditions (Lee et al. 2005).

Circular Dichroism (CD) Spectroscopy

Typically, CD gives qualitative information about kinetics of conformational transitions related to aggregation and the nature of the aggregates. Specifically, protofibril and fibril formation is accompanied by pronounced formation of β-sheet conformation that can be detected by the appearance of a characteristic absorption minimum of β-sheet at 215–218 nm in the far-UV CD spectrum. An advantage of CD over other methods is

that samples incubated at wide range of pH/temperature can be studied.

Fourier Transform Infrared (FTIR) Spectroscopy

Secondary structural transition of protein can also be studied through FTIR which is complementary to CD. In FTIR, structural transition into β-sheet conformation during amyloid formation can be detected by presence of bands near 1620 cm^{-1} (for parallel β-sheet) and 1690 cm^{-1} (for antiparallel β-sheet) in the amide I region (1,600–1,700 cm^{-1}) of IR spectrum of protein.

Congo Red Spectra and Birefringence

Dye binding protocols such as those involving Congo red and ThT are the most traditional procedures for detection of amyloids. The Congo red dye staining of amyloid fibrils, when examined under polarized light, produces an apple-green birefringence. However, Congo red spectroscopic assay is generally preferred over birefringence assay as the latter is relatively subjective and requires a positive control (known fibrillar sample). Congo red, when free in solution, gives absorbance maximum at 490 nm. An intense red color produced resulting from a shift in the wavelength of maximal absorption from 490 to around 540 nm was observed upon binding to amyloid samples.

ThT Binding Assay

The use of Thioflavin (ThT) for the detection of amyloid fibrils was first proposed by Vassar and Culling in 1959. ThT is an amphiphilic dye that forms micelles in aqueous solution. Binding of these ThT micelles to β-sheet structures of amyloid fibrils produced an enhanced fluorescence emission at 485 nm. It is worth mentioning that β-sheets are also part of native conformation of several proteins as well as of some intermediates; however, an enhanced ThT fluorescence appears to be far more specific for the β-sheets contained within amyloid fibrils. This is due to the presence of lower number and distorted nature of the sheets, in the natively β-sheeted proteins, which are unlikely to form regular binding channels for the dye molecules as in the amyloid fibrils (Krebs et al. 2005). Nevertheless, ThT is reported to give enhanced fluorescence with amorphous aggregates also (Kumar et al. 2008).

Other Fluorescence Probes

While Congo red and ThT are highly selective for fibril structure, dyes such as ANS, bis-ANS and Nile red are used as fluorescence probe for the detection of both amorphous and fibrillar aggregates. The later dyes are known to interact with hydrophobic areas of both amorphous aggregates and amyloid fibrils (Hawe et al. 2008). ANS and bis-ANS are suitable for the detection of transient aggregates which are formed in very low concentration during the early stages of protein refolding as well as aggregates formed during thermal denaturation (Finke and Jennings 2001; Grillo et al. 2001; Vetri and Militello 2005). The use of ANS in kinetic studies of fibril formation of $A\beta_{1–42}$ and SH3 domain and lysozyme has demonstrated the ability of this dye to detect the prefibrillar aggregates that are considered to be more pathogenic than mature fibrils (Ravi et al. 2014).

Intrinsic Fluorescence Spectroscopy

Fluorescence intensity (FI) and emission maxima (λ_{max}) are very sensitive to the changes in the microenvironment around intrinsic fluorophores *viz* Trp, Tyr and Phe and therefore can be used to study protein ligand interaction, folding and assembly (Zaidi et al. 2013). The intrinsic fluorescence of aromatic residues has been used to probe structural and conformational dynamics of the self-assembly of several amyloidogenic proteins such as α-synuclein, Aβ, prion, IgG light chains, etc. (Burré et al. 2014; Maji et al. 2005; Munishkina and Fink 2007).

X-ray Diffraction

This method helps in generating information about the preferred orientation of the molecules in such samples and can be applied to lower quality crystalline samples. All amyloid fibrils identified by a strong meridional reflection at ~5 Å correspond to the spacing between individual β-strands and a weak but broad equatorial reflection at ~10 Å resulting from inter-sheet spacing

perpendicular to the fibril axis in X-ray diffraction (Makin et al. 2005; Sunde et al. 1997). Additionally, equatorial reflections correspond to the spacing between the protofilaments whereas meridional reflections provide an estimate of the number of protofilaments comprising the fibril.

Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM)

Protein self-assembly into oligomers, protofibrils and mature fibrils can be visualized directly by high- resolution microscopic techniques such as transmission electron microscopy (TEM) and atomic force microscopy (AFM) up to nanometer to picometer resolution (Mastrangelo et al. 2006; Selenica et al. 2007). An additional advantage of AFM over TEM is the continuous monitoring of the oligomer and fibril formation in solution (Drolle et al. 2014).

Other Methods

Other techniques used for analysis of protein aggregates, in general, include SDS-PAGE, size exclusion chromatography, analytical ultracentrifuge, field flow fractionation, etc. These techniques are basically used for shape and size estimation, distinguishing between covalent versus non-covalent aggregates and for the quantification of soluble aggregates.

4.1.2.6 Factors Favoring Protein Aggregation

Although many different proteins and peptides have demonstrated strong potential to form aggregates with similar morphology, the individual propensity for such behaviour varies with amino acid sequence as well as the environmental conditions (Sidhu et al. 2014; Mohr et al. 2013; Dobson 2004). The following section shall discuss the variety of intrinsic (structural) as well as extrinsic (environmental) factors that are found to influence the aggregation propensity of proteins (Fig. 4.9).

Intrinsic Factors

It is generally believed that more the hydrophobicity, greater will be the tendency to form aggregation (Calamai et al. 2003). Intrinsic factors that influence the amyloid aggregation of proteins include fundamental properties of polypeptide chains such as hydrophobicity, sequence of polar and non-polar amino acid residues, charge and propensity to acquire various secondary structure elements.

The importance of amino acid sequence in determining the amyloid aggregation propensity chain can be realized from the observations that certain regions in a polypeptide chain appear to be more sensitive towards aggregation. A single mutation in these regions can drastically alter the aggregation rates when compared to the similar substitution in other regions (Lemkau et al. 2013; Camilloni and Vendruscolo 2013; Chiti et al. 2002). These findings led to the proposal of several algorithms that enable identification of local sequences with high intrinsic aggregation propensities. One such method for determining the intrinsic aggregation propensity for natively unfolded peptides like Aβ or α-synuclein has been proposed by Pawar (Pawar et al. 2005). This group has defined a parameter called intrinsic Z-score for aggregation Z_{agg} that allows comparison between aggregation propensities of different polypeptide sequences. Aggregation propensity of unfolded polypeptides as calculated using Z-score value appears to be favoured by the factors such as hydrophobic pattern and its intensity and β-sheet forming propensity but disfavoured by net charge and α-helice forming propensity.

Extrinsic Factors

In vivo, the propensity for aggregation is influenced by the similar external factors that play key roles in controlling misfolding and subsequent aggregation of proteins, *viz.* interaction with molecular chaperones, processing by proteases and efficiency of the protein quality control systems as already mentioned. Physicochemical properties such as protein concentration, pH, temperature, pressure, ionic strength and denaturants are some extrinsic factors that affect aggregate formation both *in vivo* and *in vitro*. These factors may lead to destabilization of native state and/or promote the formation of various intermediates. A brief account of them shall be discussed here.

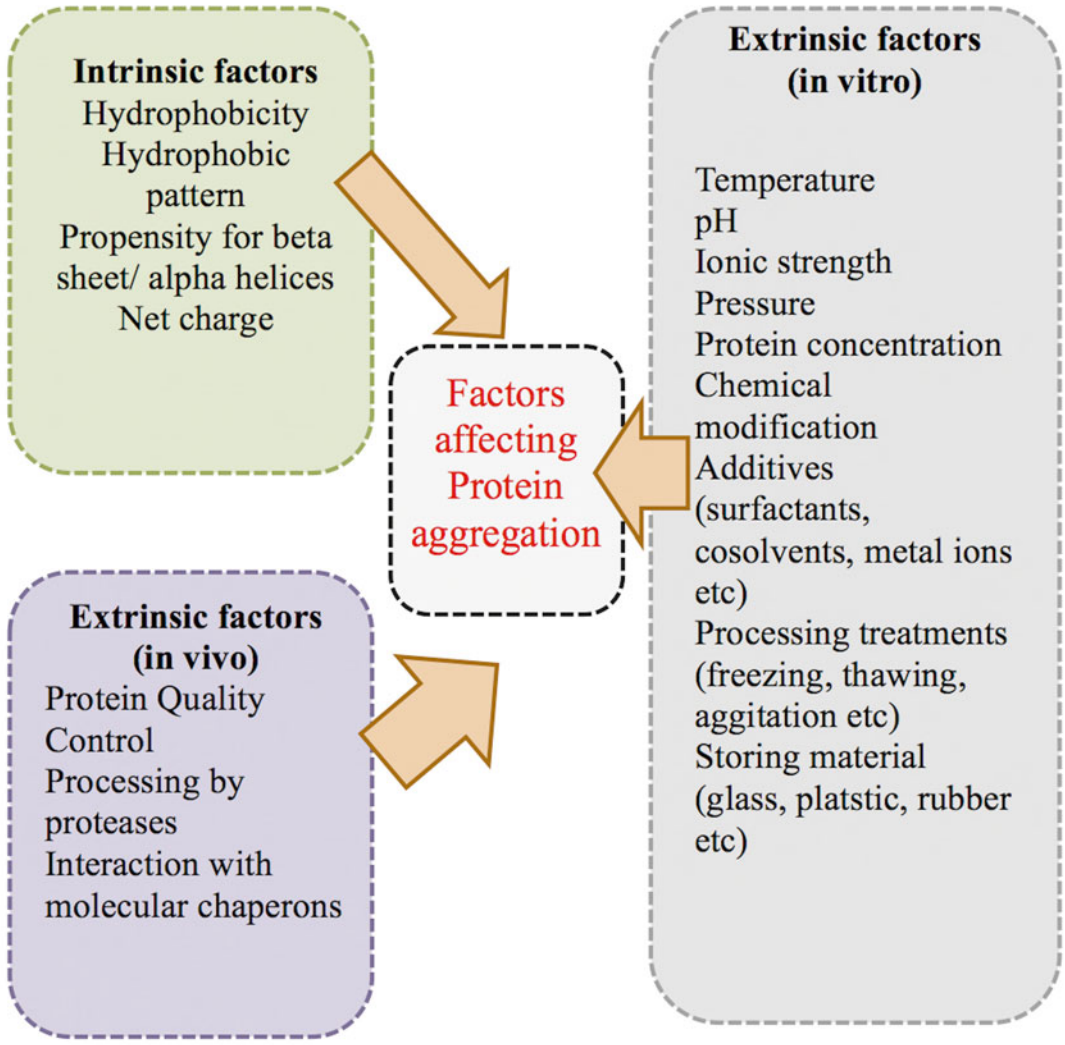

Fig. 4.9 A summary of the factors affecting protein aggregation

pH The pH of the system can have strong impact on rate of aggregation as it can potentially alter the nature as well as distribution of charges in polypeptide. Theoretically, aggregation is most likely to occur near pI, where electrostatic repulsions are minimum and so is the solubility of protein. However, experiments have demonstrated that proteins aggregate at a very slow rate around their pIs (Majhi et al. 2006). In contrast, aggregation of many proteins is actually noticed under conditions where polypeptide contains a significant net charge (Khan et al. 2014; Su and Chang 2001). An extremely acidic or alkaline pH would give high net charge to the proteins resulting in partial unfolding due to the intramolecular repulsion. However, aggregation of partially unfolded ensembles is unlikely unless the accumulated charge on protein is relieved, for example, neutralization by salts (Medda et al. 2013), surfactants (Khan et al. 2012) or exchange of protons with the surroundings (Jeppesen et al. 2010).

Ionic Strength It is another important condition that significantly impacts the aggregation behaviour of proteins. The ionic strength effect on aggregation is dependent on protein types.

If neutralization of charge by salt facilitates folding of protein or enhances its stability, then aggregation would be inhibited at low ionic strength. High salt concentration, on the other hand, would destabilize the protein, unfold it and promote aggregation. Ionic strength also considerably affects the morphology of the fibrils. Low ionic strength facilitates addition of monomers only at the growing ends of the protofibrils and prevents the interaction between axial sides due to electrostatic repulsion, thus resulting in thin and long fibrils (Nichols et al. 2002). In contrast, at high ionic strength where hydrophobic interactions predominate, binding of monomers also takes place not only at the ends of the fibrils but also along the axial sides. As a result, thick and short fibrils are formed.

Temperature Thermodynamically, the native state of protein is only marginally more stable than its denatured state. It is a general observation that unfolding of the proteins during the thermal denaturation is accompanied by their aggregation (Hansen et al. 2014; Martin et al. 2014; Vetri et al. 2007). Apart from destabilizing the protein, additional effects of temperature in promoting aggregation include lowering of activation energy, increased diffusion of the molecules, high frequency of molecular collision and enhanced hydrophobic interactions (Wang et al. 2010).

Pressure The pressure treatment may also perturb the hydrophobic interaction necessary for the native state stabilization and formation of aggregation-prone partially unfolded segments. The hydrostatic pressure has been employed as a tool for inducing the formation of amyloid aggregates in some of the pathogenic proteins such as lysozyme (De Felice et al. 2004). Aggregation induced by pressurization has also been noticed for β-lactoglobulin (Considine et al. 2007).

Protein Concentration Protein concentration is a critical factor in amyloid formation since formation of nucleus may not occur below a critical concentration of protein (Kumar and Udgaonkar 2010). Furthermore, aggregation occurring in high-concentration formulations is also a matter of concern for storage of protein (Alford et al. 2008; Shire et al. 2004).

Due to the existence of different aggregation pathways (by self-association of native monomers or initiation by partial unfolding), the increased protein concentration may exert contrasting effects on aggregation. For example, high protein concentration may increase aggregation due to increased chances of self-association. Conversely, aggregation may be decreased due to the crowding effect. This situation is generally not encountered under normal experimental conditions.

The 'macromolecular crowding' is a term which denotes the effect exerted by the total high volume occupied by the macromolecules, in intracellular compartments, on the behaviour of individual macromolecule. In accordance with this excluded volume theory, the crowding should favour self-assembly and aggregation; however, the partial unfolding of the molecules which is pre-requisite for aggregation process may be reduced (Minton 2005). Lastly, high protein concentration (when it exceeds protein's solubility limit) may cause precipitation or sometimes crystallization depending upon solution.

Surfactants Sodium dodecyl sulfate is an anionic surfactant with strong impact on protein conformation. Apart from being a protein denaturant, SDS is also well recognized for inducing amyloid aggregation in several proteins including Aβ, α-synuclein, lysozyme, bovine serum fetuin, etc. (Giehm et al. 2010; Jain et al. 2011; Rangachari et al. 2007; Zaidi et al. 2014). The amyoid-inducing action of SDS is observed below CMC of the surfactant where it exists mainly as monomers. Post-micellar concentration of SDS is reported to inhibit aggregation (Hung et al. 2010; Pertinhez et al. 2002). Also, it has been reported that aggregation induction by SDS requires development of net positive charge on protein as proteins incubated above isolelectric points (where they acquire negative charge) usually don't aggregate in the presence of SDS (Khan et al. 2012).

Chemical Modification Aggregation is also induced by chemical modification of proteins. Chemical reactions such as oxidation, hydrolysis (Van Buren et al. 2009), deamidation (Takata et al. 2008) and isomerization (Zhang et al. 2002) may destabilize protein structure thereby promoting aggregation. Besides, glycation is known to induce amyloid aggregation in BSA (Wei et al. 2009). Reduced and glycated insulin has been found to be more prone to aggregation than its non-reduced form (Alavi et al. 2013). Further, photolytic degradation of proteins which involve oxidation of aromatic residues, His, Cys and Met, is also found to induce aggregation (Mahler et al. 2009).

Miscellaneous Other factors that influence protein aggregation include organic solvents, metal ions, amino acids such as Phe, UV illumination, freezing, thawing, drying, spraying, agitation, contact materials such as glass, rubbers, plastic silicone, steel, etc. (Adler-Abramovich et al. 2012; Mahler et al. 2009; Wang 2005; Xie et al. 2011).

4.1.2.7 Inhibition of Protein Aggregation

The protein inhibition self-assembly is studied in two perspectives: developing strategies for combating protein aggregation in general (situation encountered during protein expression, refolding, manufacturing and processing of biopharmaceuticals) and identifying or designing inhibitors that specifically target amyloid aggregates (associated with several pathogenic conditions).

Inhibition of protein aggregation during the development of protein-based therapeutics is attempted by using various kinds of excipients or additives that suppress aggregation through different mechanisms such as native state stabilization, incorrectly folded conformation destabilization, protein folding increment, solvent accessibility reduction and solvent viscosity enhancement, etc. (Wang et al. 2010). The type of different additives used and their effect on protein refolding and aggregation has been extensively reviewed (Hamada et al. 2009). Denaturants such as urea and guanidine are the most conventionally used additives that assist refolding of protein and suppress aggregation.

There is a long list of compounds that are involved in protein aggregation inhibition. Amongst those, sodium dodecyl sulfate is found to inhibit aggregation at post-micellar concentration in some proteins. This action of SDS has been attributed to the increased helical propensity of polypeptide chain (Alvares et al. 2013; Giehm et al. 2010). In addition, ionic liquids such as ethyl ammonium nitrate (EAN) and non-detergent surfactants such as sulfobetaines (NDSBs) have demonstrated effective inhibition of heat and chemical-induced aggregation. Another group is of amino acids which includes Arg and its derivatives, Pro, His, Ala and imidazole is reported to exhibit potent inhibitory action over thermal aggregation of several proteins. Besides, polyamines such as putrescines, spermines and spermidines, amphiphilic polymers such as polyethylene glycol (PEGs) and polyvinylpyrrolidone (PVPs) and osmolytes such as sugars/polyols and trimethylamine N-oxides are proved to be efficient aggregation suppressors in several cases (Luo et al. 2013). Lastly, 'artificial chaperone' is another formulation that has been employed to increase the refolding efficiency of several proteins including lysozyme and serum albumins and suppress aggregation (Gull et al. 2011; Potempa et al. 2010). The term 'artificial chaperone' denotes a non-protein system with chaperone-like effect. Such systems are the combination of detergents and cyclodextrins both of which act as aggregation inhibitors and are used together to enhance refolding efficiency with reduced chances of aggregation.

Inhibition of Amyloid Formation Nature has adopted various strategies to avoid unwanted protein aggregation and amyloid formation inside a living system where local protein concentration is quite high. A study on the role of protein sequence in aggregation by Dobson et al. has revealed that low sequence identity, approximately less than 40 %, between the adjacent domains of multi-domain proteins (which form ~70 % of

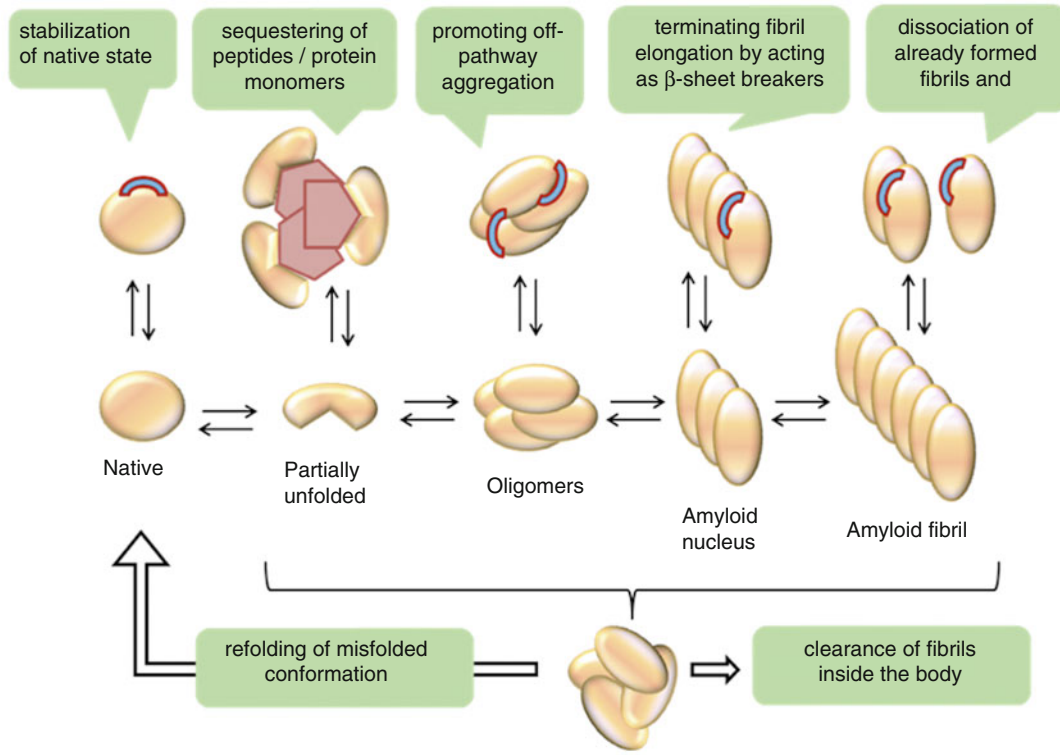

Fig. 4.10 Schematic illustration of possible strategies to inhibit amyloid fibril formation

eukaryotic proteins) could be responsible for safeguarding such proteins against co-aggregation (Wright et al. 2005). The inhibition of amyloid formation can be achieved by means of ligands (small or large) that can bind to and arrest peptides/proteins at different steps of amyloid formation pathway, viz. native state, partially unfolded state, oligomers, amyloid seeds, fibrils, etc. [Kumar et al. 2008; Kumar et al. 2009). As shown in Fig. 4.10, the possible mechanism of action of ligands which have been employed so far inhibiting fibrillization include (1) stabilization of native state, (2) sequestering of peptides/protein monomers, (3) small molecule-mediated inhibition, (4) promoting off-pathway aggregation, (5) terminating fibril elongation by acting as β-sheet breakers, (6) dissociation of already formed fibrils and (7) refolding of misfolded conformation and clearance of fibrils inside the body (Hard and Lendel 2012).

4.1.3 A Combined View of Protein Folding and Aggregation

Following its synthesis, distinct states are accessible to a protein, *viz.* native state, oligomers, disordered aggregates, prefibrillar aggregates and fibrils, etc. all having different morphology. Similar fate is encountered by proteins under *in vitro* conditions. Under specific conditions, adopted state of protein depends upon the thermodynamic stability of the various accessible conformations and their kinetic interconverison (Dobson 2004). It is now a well-established fact that most proteins are capable of undergoing fibrillogeneis under *in vitro* (denaturing) conditions. Given the fact that only a fraction of human proteins are involved in amyloid-related pathogenesis, the protein sequence and cellular machinery seems to have evolved in a manner that avoids unwanted protein aggregation. Figure 4.11 shows various

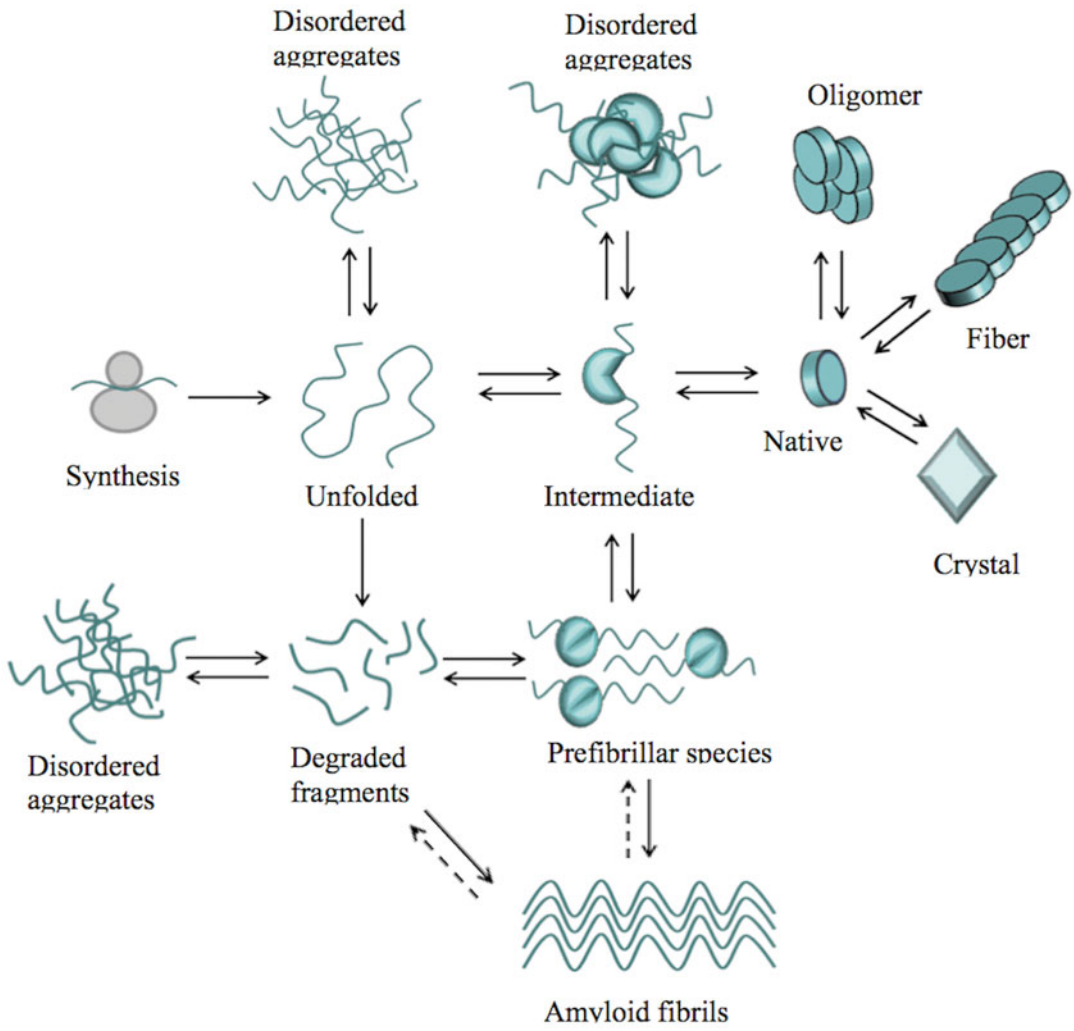

Fig. 4.11 A combined view of major structure types formed by polypeptide chains

structure types that can be formed by polypeptide chains under *in vivo* and *in vitro* conditions.

4.2 Conclusions and Future Prospects

Attempts towards understanding mechanism of protein folding have been many but still insufficient. It is a well-accepted fact that almost all globular proteins adopt partially folded conformations with properties in between the native and unfolded states. The amino acid sequence of a polypeptide determines the similarity in compactness and the amount of secondary structure between native and its intermediate states form in folding pathway. This suggests that there exist a number of intermediates from one close to the fully unfolded state to one close to the native state to one near the denatured state, and the characteristics of such intermediates may vary depending upon the protein and the experimental conditions (Fatima and Khan 2007). It is imperative that elucidating the protein folding phenomena requires characterization of all the conformational states that a protein might

acquire, because of their resemblance with *in vivo* protein folding pathway intermediates.

Recently, the heterogeneous structure of the molten globule state is reported, i.e. a portion is more organized and native like while the other is less organized. Further, protein itself determines its detailed structural properties. Thus, it will be useful to describe molten globule structure of each protein so that we can see the characteristics in common as well as also find out as to where the difference lies. In addition to folding, the misfolding and subsequent aggregation of proteins is also an important area of intensive research as it is directly related to the several pathological conditions. Furthermore, to overcome protein aggregation in commercialization of therapeutic proteins is one of the major hurdles. Although considerable progress has been made, our current understanding of protein aggregation is still incomplete. Thus, to obtain a better understanding of fibril formation mechanisms and finding methods for intervention are topics of utmost investigation. However, whatever prevention/moderate inhibition has been obtained is mostly experimental. Therefore, in the current scenario, an understanding of protein folding and misfolding mechanism is crucially required for therapeutic development against devastating diseases as well as in various biopharmaceutical processes.

More concisely, a deep insight to understand protein folding mechanisms and ways to prevent misfolding will generate new ideas for biotechnology and pharmaceutical industries, and for medical sciences.

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Protein Folding: From Normal Cellular Function to Pathophysiology

5

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Contents

Abstract

The process of protein folding is not a single-step mechanism. Newly synthesized unfolded protein molecules fold into three-dimensional (3D) conformation to execute its biological function. Any form of small inaccuracy in the folding process may direct to protein misfolding and may be damaging for cellular environment. Protein misfolding is the fundamental cause of a large number of degenerative and neurodegenerative disorders in humans that show the way to the aggregation of protein molecules. The folding of protein depends upon the biophysical characteristics of protein itself. The native structure of protein must be thermodynamically stable in all respects to exist in cells. Many proteins cannot fold themselves in appropriate way due to the viscous cellular environment that is one of the major reasons and necessitates particular kind of ubiquitous protein which is referred to as molecular chaperones. The aggregated forms of protein molecules which are not degraded accurately inside the cell are responsible for the development of amyloid-like structure that causes a range of degenerative diseases. Our investigations are limited, as far as treatment

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of protein misfolding disorders are concerned. Several studies and clinical trials have been completed to overcome the neurological and non-neurological degenerative disorders, but the success rate was not up to the mark.

Keywords
Protein folding • Neurodegenerative disorders • Protein Aggregation • Molecular chaperones • Amyloid-like structure

5.1 Introduction

Protein folding is one of the most complex processes found in the biological system. Like other organic macromolecules in the cell, protein also has its own way of synthesis as well as degradation mechanisms. The structural and functional unit of a protein and/or a polypeptide is amino acid. The process of protein synthesis starts from the heart of the eukaryotic cells, the nucleus, through a process called transcription. The process of protein manufacturing is mainly accomplished in the cytoplasm with its various components that play crucial roles for its final structure. It is necessary to achieve final globular or fibrous form of protein to perform biochemical as well as physiological function in the biological system. Moreover, most of the post-translational modifications (PTMs) are performed inside the endoplasmic reticulum (ER).

The process of protein folding is not a single-step mechanism, rather involving multiple steps and signalling cascades. The folding of protein is a process by which newly unfolded protein molecules fold into three-dimensional (3D) conformation to perform its biological function. Various types of models concerned with protein folding have been proposed in the literature. The advances in the experimental as well as computational techniques have provided the better understanding for various crucial aspects of protein folding pathways.

Proteins are molecular machines that have ability to control most of the crucial physiological functions in biological system when they are in folded state. However, the folding of protein depends upon the biophysical characteristics of protein itself (Fernandez 2013). The native structure of protein must be thermodynamically stable in all respects to exist in cells as independent to perform its role. The process is very complex in nature and is prone to many errors (Dill and MacCallum 2012). The abnormal proteins involved in diseases have one or more mutations in the structure that lead to the instability of native form or stability of misfolded form. Moreover, protein folding looks more difficult due to the presence of thousands of colliding molecules (including other proteins) in the cytoplasm, while the synthesis of protein is being accomplished (Ellis and Minton 2006). Hence, many of the proteins do not attain the final configuration and/or thermodynamic stability leading to various diseases.

All the diseases related to protein misfolding have some common chemistry in their pathology. The development of protein aggregates in biological systems is due to the instability of α-helical structure and simultaneous molecular 3D arrangement of β-sheet (Dobson 1999). The β-sheets are fashioned between the discontinuous peptide strands of amino acids. Aligned pleated structures are responsible for the linkage of strands due to the hydrogen bonding (Bennet et al. 1995). The harmful aspect of such rearrangements in the secondary structures can be observed in the form of α1-antitrypsin deficiency and cystic fibrosis (CF) when the proteins lose their function and in many neurodegenerative disorders when protein misfolding leads to development of harmful amyloids (Cohen and Kelly 2003) (Table 5.1).

5.2 Protein Misfolding

A protein molecule has to acquire 3D conformation through an intricate folding pathway to perform its function actively in biological system that depends on the linear amino acid sequence as well as the local cellular environment. Any form of small error in the folding process may lead to protein misfolding and may be harmful for cellular environment (Ellis and Pinheiro 2002; Jagannathan and Marqusee 2013). Moreover,

many proteins are unable to fold into final conformation by themselves and require molecular help in the form of another special kind of ubiquitous protein molecules, called chaperones (Kinjo and Takada 2003).

Protein folding is a complex process that has its own thermodynamic characteristics. Recognition of molecular mechanisms associated with protein assembly is really crucial in terms of gain and loss of energy when new bonds are formed and broken down. According to the energy landscape theory, folding process of protein does not follow the specific pathway; rather it is a complex self-organizing process that generally does not occur through an obligate series of intermediates but through various ways down the folding funnel. The energy of different conformation before attaining the final structure decreases with the formation of well-organized native structure. On the highest energy level, various conformations do not attain ordered structures. Moreover, the unfolded state of protein has higher degree of conformational free energy (G) and entropy (S) than that of the native form of protein. The facade of the funnel is unique for a specific polypeptide chain under specific set of conditions. Therefore, misfolded state of protein is thermodynamically unstable and unfavourable. For achieving more stability and lower energy levels, the misfolded proteins are inclined to aggregate in biological system. If the initial conformations do not follow the proper alignment and/or sequence of amino acids, it would be difficult to gain the final thermodynamically stable native form of protein. If this happens, the required physiological function will not be performed by the cell that may lead to any sort of systemic and/or neurodegenerative disorder. The process in which the native structure is disorganized is called denaturation. Because of loss of specific arrangement of amino acids, misfolded proteins are non-functional.

Protein misfolding is believed to be the basic cause of a large number of degenerative (DD) and neurodegenerative disorders (NDD) in humans including Alzheimer's disease, Huntington's disease, Creutzfeldt-Jakob disease, Parkinson's disease, cystic fibrosis and many other disorders (Table 5.1) that lead to the aggregation of protein molecules. These disorders are collectively referred to as proteopathy (protein misfolding disease) in which proteins lose their normal structure and disrupt the physiology of biological system (Luheshi et al. 2008). A degenerative disorder is a disorder in which physiology (function) and anatomy (structure) of the damaged organ(s) or tissue(s) will increasingly worsen over the passage of time like atherosclerosis, cancer and diabetes mellitus. On the other hand, neurodegenerative disorders are those in which progressive deterioration of nerve cells is evident.

Misfolding is affected by composition of amino acids as well as certain mutations in genes (Greenbaum et al. 2005; Tsubuki et al. 2003) that accelerate the process. Furthermore, misfolding of proteins either in DDs or NDDs is also associated with environmental conditions including alteration in temperature (particularly elevated temperature) (Azuaga et al. 2002), alteration in pH (Giri et al. 2007; Vetri et al. 2007; Fan et al. 2007; Carneiro et al. 2001), oxidative agents (Rakhit et al. 2002; Stewart et al. 2005, 2007), glycation (Schmitt 2006; Bouma et al. 2003), elevated hydrostatic pressure (De Felice et al. 2004; Jansen et al. 2004) and/or high ionic strength (Mantyh et al. 1993; Raghuraman and Chattopadhyay 2006).

Various degenerative diseases share common pathological behaviour of protein aggregation that lead to the formation of plaques result in damaging the normal physiology of organ function or may lead to its failure. Common pathological features in protein misfolding diseases are hyperglycemia (Messier and Gagnon 1996), oxidative stress (Stohs 1995) and hypertension (Skoog and Gustafson 2006). Alzheimer's disease can be developed as secondary disease in patients suffering from diabetes mellitus (Arvanitakis et al. 2004). Moreover, atherosclerosis is linked with increased risk of Alzheimer's disease and dementia having common pathological aspects in patients including inflammatory processes, mutations in genes (ApoE and CD36) and increased cholesterol levels (Casserly and Topol 2004).

Table 5.1 List of protein misfolding disorders in human

Disorders	Aggregated protein	References
Alexander disease	Glial fibrillary acidic protein (GFAP)	Quinlan et al. (2007)
Alzheimer's disease	Amyloids-β peptide (Aβ), τ-protein	Rasool et al. (2014), Iba et al. (2013)
Amyotrophic lateral sclerosis	Superoxide dismutase, TDP-43	Bruijn et al. (2004), Banci et al. (2008)
Cataracts	Crystallins	Surguchev and Surguchov (2010)
Cerebral β-amyloid angiopathy	Amyloids-β peptide (Aβ)	Hawkes et al. (2014)
Cystic fibrosis	CFTR protein	Brown et al. (1996), Wang and Li (2014)
Diabetes type II	Amylin (islet amyloids polypeptide: IAPP)	Pillay and Govender (2013)
Dialysis amyloidosis	β2-microglobulin	Corlin and Heegaard (2012)
Fibrinogen amyloidosis	Fibrinogen	Stangou et al. (2010)
Glaucoma	Amyloids-β peptide (Aβ)	Guo et al. (2007)
Huntington's disease	Huntingtin	Martin (1999)
Lysozyme amyloidosis	Lysozyme	Sattianayagam et al. (2012)
Medullary thyroid carcinoma	Calcitonin	Goldman and Schafer (2011)
Parkinson's disease	α-synuclein	Dobson (1999), Chowhan et al. (2014)
Retinitis pigmentosa	Rhodopsin	Mendes et al. (2010)
Serpinopathies	Serpin	Ito and Suzuki (2009)
Sickle cell disease	Hemoglobin	Stuart and Nagel (2004)
Tauopathies (multiple)	τ-protein	Wolfe (2012)
Prion diseases		
(a) Creutzfeldt-Jakob disease	Prion protein (PrP)	Obi and Nwanebu (2008)
(b) variant Creutzfeldt-Jakob disease	Prion protein (PrP)	Collinge et al. (1996)
(c) Kuru	Prion protein (PrP)	Sikorska and Liberski (2012)
(d) Fatal familial insomnia	Prion protein (PrP)	Medori and Tritschler(1993)
(e) Gertsmann-Straeussler-Scheinker syndrome	Prion protein (PrP)	Iwasaki et al. (2014)
(f) Protease-sensitive prionopathy	Prion protein (PrP)	Gambetti et al. (2011)

Protein misfolding or aggregation concept in such diseases is associated with β-sheet predisposition, low net charge as well as protein segments with hydrophobic amino acids (Linding et al. 2004). Protein segments are the precursors which facilitate the process of aggregation. Various pathways concern which precursor is formed from native protein structure including appearance of misfolded variant that suffers incomplete misfolding representing a precursor pool (Horwich and Weissman 1997; Laidman et al. 2006; Buxbaum 2004) or inappropriate or incomplete proteolysis (Stix et al. 2001). Unstructured aggregations to highly ordered fibrils (amyloids) are usually enriched in cross-β sheets (Rousseau et al. 2006).

Initially, some proteinaceous lesions were found in the brain of patients suffering from Alzheimer's disease, having few or no classical amyloid fibrils like the diffusible deposits of Aβ-protein (Wisniewski et al. 1998). It has also been observed that non-fibrillar protein aggregations (oligomers) were toxic to biological system and amyloidogenic proteins (fibrillar) may be benign in nature (Glabe 2006; Gadad et al. 2011). Normally in proteopathies, change in 3D configuration of protein leads to protein binding to itself forming an aggregated form in the cell that

is resistant to clearance from cell as well as may damage the normal physiological role of the cell and/or tissue. Those protein molecules which are relatively unstable and are present in the form of monomers like unbound protein molecules are more prone to be misfolding into abnormal configuration. Moreover, disease-causing proteins exhibit an increase in β-sheet secondary structure of that protein (Dobson 1999; Selkoe 2003).

In case of proteopathy, certain risk factors like mutation in genes (Table 5.2) are responsible for the self-assembly of protein to form aggregates. Other risk factors include destabilizing modifications in the primary sequence of amino acids, post-translational modifications (PTMs) like hyperphosphorylation, enhanced synthesis of protein molecules and decrease in its clearance from cells as well as changes in pH and/or temperature (Walker and Levine 2000; Carrell and Lomas 1997).

5.3 Degradation Systems in Cells for Misfolded Proteins

The cell has very different chemical nature in different compartments (organelles), and protein folding occurs in various distinct regions of the cell. Due to different chemical nature, multiple problems that are associated with protein folding are raised. Response to the problems by the cell in multiple ways like chaperone. expression are evident in response to the accumulation of unfolded proteins in the cell. In cytosol and nuclear region, this response is referred to as heat-shock response (HSR) while in the endoplasmic reticulum (ER) this response is called unfolded protein response (UPR). UPR is a type of intracellular signalling pathway to maintain the homeostasis of ER and is involved in responding to inflammatory, oxidative and metabolic responses (Wang and Kaufman 2012). The role of UPR is evident in various disorders like cancer, inflammatory disorders and metabolic and neurodegenerative diseases (Wang and Kaufman 2012). Moreover, multiple degrading systems may be activated including ER-associated degradation (ERAD) (Smith et al. 2011), autophagy (Nedelsky et al. 2008) as well as proteasome (Varshavsky 2012; Nedelsky et al. 2008) to degrade the misfolded proteins in the biological system. ERAD is a cellular pathway in which misfolded proteins are targeted for ubiquitination (an enzymatic, PTM process in which ubiquitin protein is bound to a substrate protein for its degradation) and following degradation process by protein-degradation complex, referred to as proteasome. The misfolded proteins are sent back to cytoplasm from ER because ubiquitin-proteasome system (UPS) is located in the cytosol. The translocation of protein from ER to cytoplasm requires a driving force that assures the direction of transport, and driving force is facilitated by ubiquitin-binding factors. Therefore, polyubiquitination is crucial for the export of substrate proteins (misfolded proteins).

ER is responsible for the coordination between biosynthesis and its secretion of protein molecules. Moreover, ER is a storage house for intracellular Ca^{2+}, where its concentration may reach up to 5 mM (Stutzmann and Mattson 2011). Release of Ca^{2+} from ER leads to either apoptotic or survival signal for the cell that is sensed by the mitochondria (Sammels et al. 2010). ER actively responds towards environmental changes including developmental as well as stress by series of signalling pathways that is referred to as UPR (Schröder and Kaufman 2005). Accumulation of misfolded protein into the lumen of ER activates the UPR that in return regulates the shape, size and various ER components according to the altering demand of protein folding not only in normal physiology but also in pathological states (Wang and Kaufman 2012).

5.4 Basic Events in Protein Misfolding

Five basic events that are observed in protein misfolding are

(a) Improper degradation
(b) Mislocalization
(c) Dominant-negative mutations
(d) Structural alterations
(e) Amyloid accumulation

Table 5.2 Basic events in protein misfolding with associated disorders

Mechanisms of protein misfolding	Disorder	References
Improper degradation	Cystic fibrosis, Gaucher's disease	Qu et al. (1997), Wang et al. (2006), Cox and Cachón-González (2012)
Mislocalization	α-antitrypsin disorder	Hidvegi et al. (2005, 2010)
Dominant-negative mutations	Epidermolysisbullosa simplex, mutations in p-53	Chamcheu et al. (2011), Freed-Pastor and Prives (2012)
Structural alterations	Alzheimer's disease, mutation in non-receptor tyrosine kinase	Rasool et al. (2014), Whitesell et al. (1994, 2012)
Amyloid accumulation	Alzheimer's disease, Parkinson's disease, Huntington's disease, cataracts	Rasool et al. (2014), Chowhan et al. (2014), Surguchev and Surguchov (2010), Chiti and Dobson (2006)

Degradation systems of protein misfolding like autophagy or ERAD sometimes become overactive and lead to the protein accumulation disorders. These systems are crucial requirements for preventing the accumulation of abnormal or non-functional misfolded proteins. Hence, improper degradation of misfolded proteins leads to more severe disorders (Table 5.2) in humans (Qu et al. 1997; Wang et al. 2006). Various proteins that are localized to specific location or compartment (organelle) must be folded and undergone through post-translational modifications in appropriate fashion in order to be trafficked well. Improper sub-cellular localization is due to the mutations in the sequences of newly synthesized protein molecules that lead to the instability. Moreover, this dysfunction is responsible for the gain of toxic function as well as inappropriate location in sub-cellular region. Hence, protein molecules are accumulated in wrong location of biological system (Hidvegi et al. 2005, 2010).

Another way of protein misfolding is referred to as dominant-negative mutations in which an abnormal protein (mutant) counteracts the function of the wild-type protein in the cell and causes disease (Table 5.2). The loss of protein activity can be observed even in heterozygous condition of an individual (Chamcheu et al. 2011). Protein structural alterations may cause dominant phenotypes in which proteins attain an unpredictable conformation that is responsible for the toxic function of protein (Rasool et al. 2014; Xu and Lindquist 1993; Xu et al. 1999). Insoluble proteins in the form of fiber aggregates are accumulated in the cell to cause a variety of disorders including neurodegenerative disorders.

Amyloidogenic proteins cause amyloid-related diseases which are categorized on the basis of toxic protein conformation in related diseases (Rasool et al. 2014; Surguchev and Surguchov 2010; Chiti and Dobson 2006). The fibrillar aggregates in the cytoplasm or at any other location in the cell contain a large number of hydrogen bonds that are highly insoluble in the cellular environment. These fibrillar aggregates are referred to as amyloids, and their development results in plaque-like arrangement (Muchowski 2002).

5.5 Molecular Chaperones

Even a minute error in the protein folding process is responsible for the structural alterations and may lead to the lethal condition for the cell. Many proteins cannot fold themselves in appropriate way due to the viscous cellular environment and require special kind of ubiquitous protein which is referred to as molecular chaperones (Kinjo and Takada 2003; Valastyan and Lindquist 2014). These proteins are responsible for the assistance

of other newly synthesized protein molecules to attain their native state for biological function and prevent the formation of misfolded conformation and/or aggregated structure that lead to various human diseases.

Chaperones are present at multiple compartments (organelles) within the cell and interact with newly synthesized protein molecules and/or polypeptides for the safer transfer from the site of synthesis to the site of physiological function. These proteins have ability to differentiate between the native and non-native states of protein molecules. Those protein molecules which are not getting their final state of physiological function are processed for degradation pathways that are the combination of molecular chaperones as well as ubiquitin proteasome system (UPS) (Berke and Paulson 2003). Both are collectively referred to as protein quality control (QC) system. This system plays an imperative role for the survival as well as function of biological system.

Various types of molecular chaperones like calnexin, BiP, calreticulin and ERp57 perform multiple functions at various locations in the cell regarding the degradation of misfolded protein molecules (Welch 2003; Swanton et al. 2003). The aggregated forms of protein molecules which are not degraded properly inside the cell are responsible for the development of amyloid-like structure that causes various degenerative diseases (DD) and in due course cell death (Berke and Paulson 2003).

Activity of molecular chaperones may be responsible for the suppression or prevention of destructive neurodegenerative disorders (NDD) in humans. Reduced levels of intracellular molecular chaperones result in an elevation of abnormal misfolded protein molecules in the cell (Welch 2003). Higher level of expression in the form of chaperone molecules can suppress the neurotoxicity due to the protein misfolding, reflecting that chaperones could be employed as therapeutic agents (Barrel et al. 2004; Taipale et al. 2013). Chemical chaperones are small molecules that have ability to stabilize the process of protein folding and stop the anomalous protein aggregation in protein misfolding diseases against chemical and thermal denaturation (Welch and Brown 1996). The examples of chemical chaperones are trehalose, tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyrate (4-PBA) (De Almeida et al. 2007). In vitro protein folding as well as synthesis requires co-solvents such as arginine and osmolytes. In biological systems, osmolytes are accumulated in the cytoplasm which results in raising the osmotic pressure against cellular water stress that lead to stabilization of protein molecules (Rajan et al. 2011). Chemical chaperones have the ability to inhibit the amyloid formation by misfolded proteins (Yoshida et al. 2002). Moreover, studies have reflected to reverse intracellular retention of various misfolded proteins by the use of chemical chaperones including α1-antitrypsin (Burrows et al. 2000), vasopressin V2 receptor (Galkin and Vekilov 2004), aquaporin-2 (Tamarappoo and Verkman 1999), P-glycoprotein and p53 (Loo and Clarke 1997), CFTR (Sato et al. 1996) and α-galactosidaseA (Chapple et al. 2001).

5.6 Therapeutic Approaches for DD and NDD

Chemical and pharmacological chaperones have been employed for the treatment of DD and NDD. But other than chaperone-based therapy, immunotherapy as well as anti-oxidative therapy has also been employed for the management of protein misfolding diseases (Chowhana et al. 2013). The idea of immunotherapy represents the increase in monoclonal antibodies against the toxic proteins which can then be incorporated into patients through injection to alleviate the burden of amyloids (Chowhana et al. 2013). Several antibodies (monoclonal) have been investigated for Alzheimer's disease (Moreth et al. 2013). Serum amyloid A protein (SAP) is a universal component of all deposits of amyloids and acts as an excellent immunogen. A therapeutic monoclonal antibody (anti-SAP) has been developed for targeting SAP (Bodin et al. 2010).

Oxidative stress has been associated with the cellular toxicity due to aggregation of abnormal protein and amyloids (Tabner et al. 2001). Reactive oxygen species (ROS) are generally

harmful for the biological system, and antioxidants act against the ROS, thus can be used as promising therapeutic agents. L-dopa-GSH, co-drugs have been synthesized that protect against the oxidative damage from mono-amine oxidase and autoxidation-mediated metabolism of dopamine. This drug can easily cross blood–brain barrier (BBB) (Cacciatore et al. 2012). An antioxidant repair enzyme, methionine sulfoxidereductase A (MsrA), has been investigated to help the inhibition of α-Syn fibrillation, ROS depletion and stopping the neurotoxicity by repairing oxidatively damaged proteins (Liu et al. 2008).

5.7 Selected Examples of Protein Misfolding Disorders

5.7.1 Degenerative (Non-neurological) Disorders (DD)

5.7.1.1 Atherosclerosis

Elevated cholesterol and blood pressure, diabetes mellitus, smoking, altered lipid metabolism and inflammatory cytokines are risk factors for atherosclerosis (Humphries and Morgan 2004). Arterial walls become thick due to the accumulation of atherosclerotic plaque that contains cholesterol, fats, lipoproteins, macrophages and calcium. About 60 % atherosclerotic lesions contain fibrillar proteins (Rocken et al. 2006). Several proteins that show misfolding *in vivo* are related to apolipoprotein family; α1-antitrypsin and β-amyloids are present in atherosclerotic lesions (Howlett and Moore 2006). Glycation and oxidation of proteins lead to the misfolding of proteins in arthrosclerosis (Howlett and Moore 2006).

5.7.1.2 Tumor Suppressor Protein (p53)

Tumor suppressor protein (p53) is a sequence-specific transcription factor that is responsible for maintaining the genome integrity. Inactivation of p53 happens due to mutation in gene sequence and has been observed in all types of human cancers (Nikolova et al. 2000). Human p53 gene mutation has been identified with high frequency in various cancers (Foster et al. 1999). The mutations in the gene affect the residues in the protein that are crucial for maintaining the structural fold of p53 to perform its proper function. Hence, abnormal p53 is synthesized by the cell with incorrect sequence of amino acids, and protein is unable to control multiplication in damaged cells; as a result, cells show uncontrolled multiplication referred to as cancer (Cho et al. 1994; Soussi and May 1996; Walker et al. 1999).

5.7.1.3 Sickle-Cell Anemia

First misfolding disease in humans was sickle-cell anemia (first inherited disease) with its known molecular mechanism (Yawn et al. 2014). In this disease, glutamic acid at sixth position of β-sheet is replaced by another amino acid called valine (single point mutation) (Ingram 1957). Mutation in the polypeptide leads to intermolecular bonding with adjacent haemoglobin molecules that result in long polymer fiber synthesis in stable form. Polymerization is responsible for the alteration in rigidity as well as shape of red blood cells (RBCs) and exhibits abnormality. Moreover, elasticity of RBCs is reduced, and extensive tissue destruction causes pain and finally anemia.

5.7.2 Neurodegenerative Disorders (NDDs)

All the neurodegenerative disorders are characterized by the formation of intra- or extra-cellular aggregates and/or ubiquitinated misfolded proteins as well as development of lesions in the brain region (Berke and Paulson 2003; Cardinale et al. (2014). Different neurodegenerative disorders have different accumulation of proteins like Alzheimer's disease (AD) is due to the τ (tau)/β--amyloid (Aβ) accumulation (Iba et al. 2013), Parkinson's disease (PD) due to α-synuclein and Huntington's disease due to huntingtin accumulation (Muchowski 2002).

5.7.2.1 Alzheimer's Disease (AD)

AD is characterized by two aggregated forms: tau tangles and amyloid-β plaques in neuronal cells. Autophagy has been concerned with the patho-

genesis of AD by its involvement with the endosomal-lysosomal system, which plays a key role in the arrangement of the amyloid-β plaques. Analyzing the function of autophagy in AD began because it was found there is an involvement of the endosomal-lysosomal system in AD pathogenesis and exclusively in Aβ-amyloidogenesis. Numerous studies have documented that the endosomal-lysosomal pathway is a central controller or regulator of amyloid precursor protein (APP) processing (Grbovic et al. 2003; Pasternak et al. 2003).

5.7.2.2 Parkinson's Disease (PD)

In Parkinson's disease (PD), misfolded/abnormal proteins are accumulated in intracellular space of neurons (Forloni et al. 2002). PD is characterized by postural instability, muscular rigidity and resting tremor and as far as pathology is concerned, degeneration of dopaminergic neurons as well as deposition of intra-cytoplasmic inclusion bodies known as Lewy bodies in the neurons. The accumulation of α-synuclein (Chowhan et al. 2014) leads to oxidative stress (Osterova-Golts et al. 2000; Souza et al. 2000), mitochondrial malfunctioning (Hsu et al. 2000) and degradation of caspase (Da Costa et al. 2000).

5.7.2.3 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis is a neurodegenerative disorder in which death of neurons leads to muscular paralysis (Bruijn et al. 2004). It has been reported that ALS exhibits protein aggregates in the motor neurons of the patients. The SOD aggregates are formed due to the destabilization of protein. About 100 single point mutations (Fig. 5.1) have been documented in the SOD1 (copper-zinc superoxide dismutase 1) to be associated with familial form (fALS) (http://alsod.iop.kcl.ac.uk/Als/index.aspx; Banci et al. 2008). These mutations are linked with whole protein structure. Genetic association on the basis of mutations between fALS and gene of SOD1 was first reported in 1993 (Rosen 1993). SOD1 mutants exhibit pathogenicity due to the gain of toxic functions but do not lose normal function in the biological system (Mulligan and Chakrabartty 2013). Cytogenetically the *SOD1* gene is located on chromosome 21.

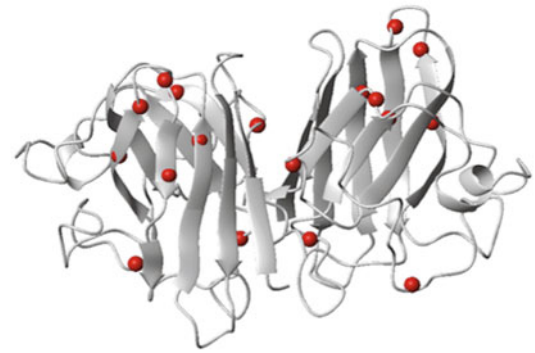

Fig. 5.1 Structure of SOD1 with location of investigated mutations (As *red* spheres) (Adapted from Banci et al. 2008)

The aggregation process regarding SOD1 has been observed related to the temperature as well as the redox conditions of the cell (Banci et al. 2008). Oxidative stress is considered to be involved in the formation of disulfide bonds; on the other hand, reducing conditions are expected to prevent the process of aggregation (Ferri et al. 2006). It has been suggested that non-physiological disulfide bonds between cysteine residues (intermolecular) at positions 6 and 111 of mutant form of SOD1 is imperative for the formation of high molecular mass aggregation in cells (Niwa et al. 2007). Investigation has revealed that mutations in SOD are responsible for the alteration in local hydrophobic structure leading to the ALS disorder (Rajasekaran et al. 2012). Moreover, it has been suggested that carbon distribution analysis program is effective in modifications as well as identification of mutations in protein sequences (Rajasekaran et al. 2012).

5.7.2.4 Creutzfeldt-Jakob Disease (CJD)

Creutzfeldt-Jakob disease is an autosomal inherited disorder and considered as the most common prion disease in humans. The sporadic form is due to the endogenous formation of prion in the body. Variant CJD is considered to be spread due to the conformational alterations in the protein molecule in which alpha helices of protein (PrP) are converted into β-sheet, resulting in aggregation and misfolding (Prusiner 2001; Obi and

Table 5.3 List of mutations in various protein misfolding diseases

Diseases	Misfolded location	Proteins involved	References
Alzheimer's disease (AD)	AD1, 2, 3 and 4, preselinin 1 and 2, Tau	Amyloids precursor protein (APP)	Games et al. (1995)
Cancer	G245, R175, R282, R248, R249, R273	p53	Nikolova et al. (2000)
Cystic fibrosis	ΔF508	CFTR	Brown et al. (1996)
Fabry	Q279E, R301Q	α-Galactosidase A	Fan et al. (1999)
Huntington's disease (HD)	HTT	Huntingtin	Martin (1999)
Parkinson's disease (PD)	A30P, A53T	α-synuclein	Dobson (1999)
Spinocerebellar ataxia (SCA)	SCA	Ataxin	Martin (1999)
α-antitrypsin disorder	D342K	α-antitrypsin	Sato et al. (1996)

Nwanebu 2008; A.D.A.M. Medical Encyclopedia 2013) (Table 5.3).

5.8 Conclusion

Our knowledge to treat various diseases particularly related to proteopathy is limited. Scientists have tried their level best to explore the pathology of disorders associated with protein misfolding, but unfortunately the information is limited. Various protein degradation processes have been investigated in different diseases and helped us to understand the aetiology as well as pathology of protein misfolding disorders and gave insight into the management of these disorders. Treatment of degenerative diseases both neurological and non-neurological that share the formation of amyloids with the help of monoclonal antibodies will serve as an ideal strategy because (SAP) is a universal component of all deposits of amyloids and act as an excellent immunogen. As far as therapeutic approach is concerned, immunotherapy and anti-oxidative therapy seems to be promising for the management of degenerative diseases.

5.9 Future Prospective

Protein misfolding disorders are associated with complex hidden parameters which make our understanding of the disease more difficult. Investigation of neurodegenerative disorders at molecular level has opened new windows to explore the molecular chemistry behind the protein misfolding. Management of disorders associated with protein misfolding will be more convenient with the advancement of biochemical as well as molecular understanding of diseases. Treatment of misfolded protein may be done with the help of molecular chaperones in future with more precise targeting strategies. The proper treatment of non-neurological diseases at proper time in patients can help to avoid the NDD because these become secondary disorders when non-neurological ones are not treated well. Better management of non-neurological diseases will help the patients to live with better lifestyle. Online databases related to proteins will be helpful to study the normal as well as abnormal structure of proteins. Not only the experimental approach but also the computational approach (*in silico*) will be helpful for the management of proteinopathies.

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Protein Misfolding Diseases: In Perspective of Gain and Loss of Function

6

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Contents

Abstract

Misfolding of proteins is quite a familiar occurrence within the cell. The most common reason for this can be either genetic variations – acquired or inherited or some post-translational modifications of proteins. These if left unchecked may create havoc within the cells. Protein folding is guided by universal driving force for lower free energy state than the unfolded one. But there are several proteopathies – protein misfolding diseases as discussed below in detail that have changed the cellular functions tremendously leading to cell death. In some it is toxic gain of function that means the misfolded and aggregated protein although loses its native form or function but develops the tendency of turning other proteins into aggregates by means of intermolecular interaction as exampled best in well-known proteopathies like Parkinson's disease, Alzheimer's disease, etc. Another situation is where protein simply loses its natural role and keeps on getting accumulated inside cells as aggregates choking them of their normal routine processes, e.g. Alpha 1 antiproteinase deficiency. The story down from here will try to shed light on how a single mutation can influence protein structure, alter its folding pattern and functioning and how it turns into a disease. Further, this chapter will shed some light on what are the future prospects of going back to normal/native state.

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Protein misfolding • Insoluble aggregates • Neurodegenerative diseases • Proteopathies • Amyloid formation

6.1 Introduction

Protein folding process starts as so-called nucleation-condensation mechanism where basic interactions develop between residues in the first stage of folding which further progresses via number of transition states and interatomic interactions and culminate into a well-folded native protein conformation having its own unique three-dimensional structure and function. Protein folding process is pictured as a topological landscape representing different energy levels. Here the lowest energy level is the minima in the energy landscape and every protein achieves its native state only when it goes to this minimum free energy level (Dill 1985).

The folding process has been deciphered mostly by study of small peptides ranging in amino acid length of 60–100, the reason being that small proteins fold into native structures thus lacking the presence of vastly populated intermediates. As the number of residues in the small proteins is less, their individual contribution in the interatomic interactions and the overall folding process can be easily ascertained. A specific mutation can be incorporated in the corresponding gene and thus into the protein product followed by folding studies which clearly will show whether it has any effect on its folding pattern. This has provided some useful leads in the mechanism of folding, particularly the formation of transition states which are the critical levels of energy surfaces through which all molecules have to pass to result in native structural fold. Almost all the literature on protein folding suggests that the protein folding pathway goes via formation of a folding nucleus around which the remaining part of the protein folds.

The core or the folding nucleus which appears during the folding process gains native-like environment as a result of favourable interactions between the residues. These interactions among the main residues force the amino acid sequence to adopt an elementary native-like structural design. The most essential requirement for correct folding pattern to initiate is proper hydrophobic and polar interactions between respective residues which start the nucleation process and the structure favourably becomes correctly packed in. Achieving the correct topology will automatically generate native fold during the very first phase of folding pattern. These key interactions among the residues are prime in formation of stable globular structure which in turn acts as a quality control mechanism to avoid misfolding to initiate. For smaller proteins the folding pathway involves mostly two state mechanisms, but for larger proteins folding pattern is much complicated process and involves appearance of intermediate or transition states between the fully folded and unfolded states. Various experimental evidences are available which show that during the folding process of protein chain some portions tend to develop native-like conformations while others are yet to attain the folded state. Another situation is that the protein sequence develops some non-native interactions the result of which could be generation of some transient misfolded state (Capaldi et al. 2002).

It can therefore be visualized that for larger proteins the folding takes place in diverse segments or domains simultaneously and independently as packets of folding states. This scenario ensures correct key interactions in these folding packets which by further specific interactions amongst each other develop into correctly folded compact three-dimensional protein structure. The final form of correct native structure is only achieved when all these folded modules interact correctly within and in between the domains and ultimate cooperative folding step is completed only when all these modules come close to each other in their exceptional close-packed arrangement excluding the water molecules from the core and make the structure highly stable and compact (Cheung et al. 2002). This mechanism is highly appreciable in that for larger protein smaller segments will form correctly folded states following two state mechanisms and then these modules will compact themselves via

specific interactions into compact structures. This can further explain formation of macromolecular structures involving multiple protein complexes and even nucleic acids. This mechanism rightly explains how large complexes like ribosomes and proteasomes are efficiently compacted into their correct conformations.

6.2 Protein Packaging Inside the Cells – Preventing Misfolding

Within the cell ribosomes are the prime machinery where new proteins are continuously synthesized using the genetic information present in the DNA which is carried to ribosomes in the form of mRNA and for some proteins the folding process starts right there during the synthesis process itself which is being referred to as co-translational folding. Others fold in the cytoplasm only after their synthesis is complete and for some proteins folding process starts while being trafficked and translocated to ER or mitochondria. The fundamentals of protein folding developed from various in vitro studies will always remain the same although the local cellular environment also has some bearing. Within the local environment some of the polypeptide regions which are otherwise buried in the native state if exposed will lead to some unfavourable interactions with other molecules. This explains that during some folding pathways transient non-native states develop in order to hide the regions of protein chain which otherwise will lead to aggregates on interaction with other molecules, e.g. the hydrophobic patches. But within the cells there is a fullproof mechanism of proper protein folding where such interactions are not allowed to happen anywhere before the protein folding process is completed (Hartl and Hayer-Hartl 2002).

Next comes the role of molecular chaperones which are present in almost all cells and within cellular compartments and these provide the important mechanism to protect against aggregation. Although chaperones have similar general roles, when examined individually each has a specific role to play in correcting misfolding. Molecular chaperones have different roles to play – some chaperones bind to protein chains even that early when they are being synthesized on ribosomes and this binding usually can be nonspecific to hydrophobic patches to prevent their interaction with other molecules and resulting aggregation. Some other chaperones have been found to be involved at later stages of the folding process. Bacterial chaperonin GroES and its co-chaperone GroEL are the most widely studied chaperones and they have made the functioning of chaperones lot more clear. Besides molecular chaperones, other types of folding catalysts that accelerate steps in the folding process which can otherwise be very slow include protein disulphide isomerases – that enhance the rate of formation and reorganization of disulphide bonds within proteins and peptidylprolyl isomerases – that increase the rate of cis/trans isomerization of peptide bonds involving proline residues.

Most of the proteins which are synthesized in the eukaryotic cells are secretory proteins and are transported outside the cells via secretory vesicles. These proteins still when being synthesized on ribosomes as nascent chains are translocated into ER where folding takes place and then sent to Golgi apparatus where they are then finally packaged for their respective destinations. In ER, a number of molecular catalysts and chaperones are present that do the folding job; in addition the proteins thus folded have to undergo a quality control check before being packaged for their destinations. This quality control check has tremendous importance in terms of controlling misfolding as it ensures whatever is transported outside the cellular environment is in its correct folded and functional state. There are almost no chaperones present outside the cells, except the recently discovered clusterin chaperone.

Misfolding is an inherent part of the folding mechanism owing to the complexity of the process. During the folding process there always stands a chance that some proteins may not achieve their native three-dimensional fold and in that case nature has already provided a back up mechanism in the form of PQC, i.e. protein quality control system that has to degrade all the wrongly folded proteins. PQS system consists of two components – molecular chaperones and the

UPS, i.e. ubiquitin protease system – and again this mechanism does the job with very high fidelity (Lakshmi et al. 2013).

The mechanism of molecular chaperones is quite clear in that they influence the kinetics of the folding process to enhance the efficiency of folding and inhibit any event that may lead to production of some unwanted products, e.g. aggregates. The chaperones set their target on unfolded and partially folded proteins. These in particular have shown hydrophobic patches which are sequestered in the center of the folded proteins. These hydrophobic regions present in the proteins have the ability to be reversibly bound and free from the chaperones. Now the chaperones owing to having an ATPase domain can induce and regulate conformational changes and can switch the molecule between low and high binding affinity states (Frydman 2001).

In addition to this, the degradative part of PQC system may capture the misfolded proteins and eliminate them. The constituent proteases of PQC system have a specialized mechanism whereby these folded proteins don't gain access to the proteolytic active sites present inside the cavities of these proteases (Groll and Clausen 2003). Before they are injected into the proteolytic active sites of proteases, proteins must first be fully unfolded and then they are processively degraded into smaller peptides. Proteasome degrades its target proteins by ubiquitin-tagging system whereas the mitochondrial PQC proteases choose its target proteins using chaperone domains.

Under in vivo conditions, oligomer and aggregate formation occurs only when significant concentration of misfolded proteins arises. Accumulation of aggregated proteins inside the cells will lead to formation of amyloid-like structures which ultimately may cause various degenerative disorders forcing cell death. Usually the PQC system has the ability to eliminate defective ribosomal products which have been synthesized as a result of errors in translation or post-translational processes or proteins which have arisen due to genetic changes in the respective individual as well as proteins that have been modified by covalent modifications especially by ROS – reactive oxygen species.

There may develop disturbances in PQC system itself owing to the following reasons: overloading with aberrantly folded variant proteins, environmental stress or genetic changes in the components of PQC system. All these changes can trigger disease processes. The consequences of the misfolding may be varied subjective to the following reasons: the nature of the protein that has been misfolded, the cellular compartment where misfolded protein is located, the effectiveness of the folding and degradation mechanism, cell and environment conditions, interaction of genetic factors, etc. A number of diseases like from early-age inborn error of metabolism to late-age neurodegenerative diseases are in fact protein misfolding diseases and thus are referred to as conformational diseases (Stefani 2004).

The most obvious reason of protein misfolding is induction of a mutation in the corresponding gene as a result of which there is wrong incorporation of amino acids and thus improper folding. This variation in the genetic code has a direct bearing on the abnormalities in the resulting protein folding which could be anything like decreased presence of specific protein that was never folded into functional state (loss-of-function) or accumulation of incorrectly folded protein inside or outside of the cells as these misfolded proteins are usually insoluble and tend to form aggregates (gain-of-function) – these situations can always become a start point for various diseases. The insoluble nature of protein aggregates is due to their high tendency for intermolecular hydrogen bonds which result in attaining febrile structure. This is what is called the amyloid fibrils and as they tend to accumulate they form amyloid plaques (Muchowski 2002).

6.2.1 Protein Misfolding/ Aggregation May Lead to Gain of Function

The protein misfolding is widely deliberated upon as it has become one of the reasons for various diseases. These disease states usually have protein aggregates present inside or outside the cells. Aggregated masses which develop as a result of

misfolding and structural changes exert toxic gain-of-function situation on the cell. It has been widely accepted now that these protein aggregate forms inhibit proteasome functioning and can thus lead to neurotoxicity. As the individual ages the overload of misfolded proteins increases and molecular chaperones no longer are able to rectify these misfolded proteins with required fidelity resulting in accumulation of more aggregates; as a result, phenotypically silent mutations are not repaired and they accumulate over time and cause disease (Csermely 2001).

Protein misfolding probability in cells is quite good as some wrong interactions develop between folding intermediates which are otherwise normal constituents of folding pathways, as a result of which supramolecular structures are formed which kind of create blocks within or outside the cells. The factors which facilitate protein aggregation are incomplete unfolding during various processes, be it oxidative or thermal stress, modified primary structure of proteins, genetic changes at DNA or RNA level, post-translational changes, etc. The design of these aggregates is usually planned and prearranged, i.e. as amyloid fibrils or formless. These protein aggregates are mostly insoluble but are stable inside the cells. Protein misfolding is pictured as 'seeding nucleation' model (Soto et al. 2006). During the early stage of this process misfolding is thermodynamically unfavourable and progresses very slowly but once the least rigid oligomeric unit is formed it will grow exponentially and at a more rapid speed. Thus seeding-nucleation model of polymerization has two kinetic phases. The first process is very slow referred to as lag phase where small quantities of misfolded-oligomeric structures then act as starting points or seeds for further misfolding. Next is the elongation process that starts from these nuclei which then grow fast into polymers. Experimentally if preformed seed nuclei are added it will further reduce the lag phase and exponential growth of polymers will be much faster. Thus it clearly depicts that to propagate misfolding exponentially, oligomers are perhaps the best seeds. But in a broader perspective large fibrils can propagate polymerization better compared to smaller ones because they are resistant to biological clearance.

Amyloid formation which represents an aggregated mass is a uniform trait in a number of proteopathies. Further after initiation of aggregates plaque formation takes place. These plaques contain good quantity of amyloid fibrils consisting of long β-pleated sheets with some β-strands running vertical to main fibre axis. These structures can very well be visualized by transmission electron microscopy (TEM) or atomic force microscopy (AFM). This experimentation has shown that fibrils are made of 2–6 protofilaments, the diameter of each being 2–5 nm (Serpell et al. 2000b). Some of these protofilaments are twisted in the form of ropes 7–13 nm wide or exist as long ribbons 2–5 nm thick and 30 nm wide. Sometimes staining techniques have been used to view these fibrils, e.g. by using stains like congo red (CR) or thioflavin T (ThT).

Main interactions which stabilize the central structure of these fibrils are hydrogen bonds present in polypeptide backbone chain. As this mechanism of hydrogen bonding is common in all kinds polypeptide main chains so irrespective of the protein type the fibrils it forms will be similar (Dobson 1999). This is in contradiction to general native protein structure which is highly specific and individualistic. The tendency to produce amyloid structures although is mostly general but will vary depending upon the circumstances the protein sequences stay in. Some of the living systems have made use of this generic ability to form amyloid structures for specific purposes. In some organisms these aggregated structures are normal and functional. However as yet this phenomenon is not clear how nature decides where these aggregates are abnormal and where beneficial. In higher animals and humans these are almost always precarious. Exception is one prion protein although responsible for prion disease in mammals has been found to be beneficial in certain organisms.

6.2.1.1 Toxic Amyloid Formation – A Deadly Cascade

The neurodegenerative diseases are mostly chronic and progressive as these involve degeneration of neurons in various brain systems. Brain lesion formation is present in every such disease and the reason for this is aggregate formation

inside or outside the cells plus presence of ubiquitinated proteins. A number of proteins like α-synuclein, tau and β-amyloid (Aβ) and prion protein are linked with the neurodegenerative diseases like Alzheimer's (AD), Parkinson's (PD) and prion diseases.

Alzheimer's Disease

AD starts around the age of 65 years and with passing of every 5 years the incidence doubles and the prevalence is about 1 %. The familial forms of the disease are documented less frequently as right diagnosis is difficult plus reconstituting family tree is not always feasible. A large number of genetic determinants may have a possible role in disease development and the family history of dementia is a most prone threat in AD (Richard and Amouyel 2001). AD presents as extracellular accumulation of Aβ amyloid plaques and neurofibrillary tangles (NFT) in the brain. It has been strongly supported that both proteins are responsible for the neurotoxicity. Aβ originates by the proteolytic processing of βAPP – β-amyloid precursor protein. Polymers of the Aβ are deposited in the extracellular and then develop to amyloid plaques. β-amyloid peptide which is of 42 amino acid length is generated from APP-amyloid precursor protein a normal membrane precursor protein. β-secretase proteolytically cleaves APP and generates Aβ-42 instead of normal Aβ-40 peptide fragment. This new altered product Aβ-42 is amyloidogenic. Under normal physiological circumstances Aβ-42 when produced in small quantities undergoes cellular degradation but under some situations leads to extracellular aggregates that finally end up as amyloid plaques. A tremendous increase in neurotoxicity has been observed when Aβ-42 forms dimers and oligomers (Higuchi 2005). Neurofibrillary tangles represent a mass of paired, helically wound protein filaments (PHFs) which are present in the cytoplasm of neurons and neuritic processes. It has been shown that PHFs in neurofibrillary tangles are microtubule-associated protein-tau. Tau proteins are essential to microtubule stabilization as they cross-link the adjacent microtubules. Tau proteins are explained as a group of cytoplasmic proteins in which alternate splicing has taken place. These have three or four microtubule-binding domains which coassemble with tubulin onto microtubules and make these structures stable (Lee et al. 2001). A number of evidences support the fact that tau mislocalization and its misfolding is primarily initiated by Aβ.

Parkinson's Disease

PD is a slowly progressing disease whose pathology involves two distinguishing features, i.e. presence of Lewy bodies in the cytosol of brain cells and loss of dopaminergic neurons in the substantia nigra. It is not yet clear how this happens and how these neuronal cells are lost. For PD no specific reasons have been established although it may happen sporadically, under the influence of environmental agents or genetic risk factors may be involved. A most possible reason of PD is over-expression of the alpha-synuclein (SYN) gene. A heritable form also arise owing to some point mutations. PD develops as toxic gain-of-function pathogenesis. In this scenario the aggregates that develop in cytosol of neuronal cells can be either a wild-type or α-synuclein variant or constituents of the ubiquitin-proteasome system or other accumulated cellular proteins. This intracellular accumulation of α-synuclein leads to oxidative stress, mitochondrial dysfunction and caspase degradation emphasized by mutations connected with familial parkinsonism. SYN has good sequence homology in vertebrates showing its significance in these organisms. It has been shown to inhibit phospholipase D2 – a plasma membrane protein that converts the phosphatidyl choline to phosphatidic acid and leads to reorganization of cytoskeletal structures and a well-directed movement of vesicles. SYN binds to tau – a microtubule-associated protein leading to its phosphorylation through protein kinase A. SYN is also dopamine (DA) transporter regulator. It also modulates activity of tyrosine hydroxylase, a rate-limiting enzyme in DA synthesis (Perez and Zigmond 2000). Thus SYN is involved in regulation of transmission dopaminergic neurons and vesicular traffic. Therefore when SYN forms aggregates

because of mutations or other changes end result is loss of dopaminergic neurons which is a characteristic of PD.

In terms of protein folding, SYN is envisaged to have a random coil structure but upon interaction with specific phospholipids there is a profound increase in its α-helical content (Jo et al. 2000). SYN not only associates with membranes but other cellular proteins also which makes its physiological functions more diverse. This conformational flexibility of SYN can expose its hydrophobic patches (especially in central NAC peptide) and increase chances of SYN aggregate formation. In vitro studies have shown to a remarkable extent that SYN forms fibrils with a high β-sheet content which are like the filaments of Lewy bodies (Serpell et al. 2000a), PD-associated mutations and C-terminal truncation – a post-transductional modification – nitration and oxidation may lead to an increased fibrillation rate.

Early start recessive forms of PD have mutations in following genes – *PARKIN*, *UCH-L1*, *DJ-1* or *PINK1*. These genes express proteins that have role in turnover of α-synuclein ubiquitination. Mutation in these genes leads to loss of PQC functions which in turn leads to aggregation of α-synuclein and oxidative stress causes mitochondrial dysfunction. These facts are further validated by post-mortem analysis of sporadic PD samples that show loss of ubiquitin-proteasomal activity. These also show signs of more oxidative stress, which clearly shows that both sporadic and familial PD thereby develop through same fundamental mechanisms (Moore et al. 2005).

The Transmissible Spongiform Encephalopathies

The transmissible spongiform encephalopathies (TSEs) is not an individual disease but a cluster of rare neurodegenerative diseases which includes chronic wasting disease (CWD) in deer and elk, scrapie in sheep and most common bovine spongiform encephalopathy (BSE) in cattle. In humans TSE presents as

1. Creutzfeldt Jakob disease (CJD) – sporadic origin
2. Mutated prion protein – heritable
3. Acquired, due to ingestion or inoculation of materials contaminated with TSE

The TSEs find representation within the spectrum protein misfolding disease because the key pathogenic incident is change in host prion protein (PrP). PrP – a mammalian glycoprotein has signal peptide and glycophosphatidyl inositol (GPI) membrane anchor sequences which are cleaved to generate about 208 amino acid chain. PrP is first glycosylated at two N-linked glycosylation sites and then is attached to the cell surface through GPI anchor. PrP^{c} loses its wild-type α helices and gets converted to β-sheet-dominant PrP^{Sc} as a result of conformational changes that ultimately leads to misfolding and aggregation. Prion protein disease symptoms include progressive insomnia, dementia and autonomic dysfunction. It is the ability of PrP^{Sc} – the infectious scrapie protein (PrP^{Sc}) – that leads to conversion of normal cellular PrP^{c} into disease-causing PrP^{Sc} in case of infectious prion disease. The normal function of prion protein is not known. PrP^{c} has good helical content and is protease sensitive and soluble whereas PrP^{Sc} – disease-causing transmissible isoform – is insoluble and protease resistant. Thus more and more PrP^{Sc} is generated with high β sheet content. Experimentally it has been shown that normal PrP^{c} has a neuroprotective function but the defective prion PrP^{Sc} changes conformation of various proteins and induces aggregate formation (Zanata et al. 2002).

Polyglutamine Disease

Polyglutamine disease (PGD), as the name suggests, is a human disorder where disorder glutamine is repeated at various regions in the polypeptide chain and that becomes a reason for its misfolding. The coding region has several trinucleotide repeats coding for phenylalanine which ultimately after translation present as polyglutamine (polyQ) tracts. This unusually long string of glutamine residues in protein product leads it to form large detergent-insoluble aggregates with in nucleus and cytoplasm. This will ultimately push for deterioration of

concerned neuron. PolyQ diseases thus have amyloid-like deposits of the defective protein in the central nervous system (CNS) like most age-related neurodegenerative disorders. The expanded form of polyQ proteins are prone to aggregate and more the expansion, more robust the aggregation. The longer the repeats, the more widespread will be neuronal degeneration and accordingly varies the disease severity in terms of the extent of CNS pathology and age of onset of symptoms. The polyglutamine (polyQ) disease represents as one of the most important cause of heritable neurodegeneration and comprises of at least nine disorders (Todi et al. 2007).

Among these Huntington disease (HD) is mainly prevalent of all the neurodegenerative disorders with an extended repeat poly (8) length. In HD patients, the repeat length may be more than 55, and accordingly the degree of aggregation will increase as the length of the repeats increases. Thus the patients with longer repeats will have early onset of disease than those with less number of glutamine repeats (Zoghbi and Botas 2002). The glutamine repeats have the propensity to self-aggregate with an unknown number of other amino acid strings in cellular proteins like α-synuclein and amyloid β-peptide, which are considered pathogenic in AD and PD, respectively.

Huntingtin aggregation exerts its derangements as gain-of-toxicity effect. It has been experimentally proven that mutant Huntingtin protein aggregate with other glumatine-rich proteins and make them defective and non-functional; this was proved by using transcription factor CREB binding protein which is rendered non-functional. Proteosome activity is also affected by Huntingtin aggregation. Huntingtin protein is proteolytically targeted inside brain cells as ubiquitinated and inclusion-localized Huntingtin has been observed to be present but this proteasome degradation is ineffective (DiFiglia et al. 1997). Huntingtin co-aggregation leads to proteosome dysfunction as the constituents of the proteosome machinery get trapped into inclusion bodies (Bence et al. 2001).

The exact changed conformations which proteins adopt inside the cells are difficult to identify because how glutamine repeat stretches lead to Huntingtin aggregation and how cellular functions are affected has not been fully established. The experimentation in this regard is deficient as no assay has been yet established for confirmation and quantification of Huntingtin aggregates in cells. The only procedures available are ex situ studies such as SDS-solubility assays or antibodies designed for conformation-specific epitopes. Till date the exact picture of Huntingtin aggregates whether inside or outside inclusion bodies is not clear therefore it can't be established what actually and how the cells functioning is defective.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is another neurodegenerative disorder wherein Cu, Zn superoxide dismutase gene (*SOD1*) is mutated. Here the misfolded SOD1 protein aggregates and leads to toxic gain-of-function. Normally the function of SOD1 is to control cellular oxidative damage which is mediated by catalyzing the dismutation of the superoxide radicals to hydrogen peroxide and oxygen. The pathophysiology in this disorder therefore is aggravated oxidative damage arising due to enzymatic haploinsufficiency. The experimental evidence in some reports is different because induced decrease in activity of SOD1 does not have the same effects as ALS phenotype in animal models (Bruijn et al. 1998). The variant protein can have several consequences such as loss of protein function due to co-aggregation with other aggregates, abnormal chemistry of the Cu and Zn sites, defective proteosome which has high misfolded protein content, reduction in molecular chaperones plus the mitochondrion and peroxisome functions can also get affected.

Autosomal Dominant Familial Neurohypophyseal Diabetes Insipidus

Autosomal dominant familial neurohypophyseal diabetes insipidus (FNDI) also has misfolded proteins present in ER which exert their effects as toxic gain-of-function pathogenesis. Congenital nephrogenic DI (NDI or CNDI) and FNDI are two hereditary forms of familial DI. These two

forms constitute less than 10 % of all DI cases which have been reported in clinical practice (Fujiwara and Bichet 2005). NDI is X-linked characterized by renal insensitiveness to the antidiuretic effects of arginine vasopressin (AVP). One of the reasons for NDI is autosomal dominant (or recessive) form due to mutations in the gene coding for the renal aquaporin 2 water channel (the AQP2 gene; Entrez GeneID 359) or mutations in the gene coding for V2 receptor (the AVPR2 gene; Entrez GeneID 554). FNDI is due to autosomal dominant inheritance (adFNDI) and appears to be largely penetrant, if not completely (Christensen et al. 2004a). AVP defect has mostly dominant inheritance as other isolated deficiencies of pituitary hormones (e.g. sexual ateliosis, or isolated growth hormone deficiency) are recessive. The dominant AVP defective form has been widely studied in terms of its protein misfolding, aggregation and neurodegeneration implications. The common symptoms of FNDI are polyuria and polydipsia which arise due to decreased neurosecretion of the antidiuretic hormone, AVP (Christensen et al. 2003), which is otherwise normally formed by the magnocellular neurons. These specialized neurons are situated in the paraventricular and supraoptic nuclei of the hypothalamus and form the neurohypophysis by their long extensions projecting down through the diaphragma sellae. Till date 50 different mutations in the *AVP* gene have been linked with FNDI (Christensen et al. 2004b; Siggaard et al. 2005). The variant precursor hormone when synthesized is not properly folded in ER but is rather retained by PQC system resulting in cytotoxic buildup of this protein in the neurons.

A number of hypotheses have been proposed for explaining the molecular mechanisms underlying the development of the FNDI, and the basis for all of them are the unique clinical, pathological and biochemical characteristics of the disease. There are a number of mutations or variations involved in this disease but the clinical phenotype has minimal variation. The possible reason for this is that all these mutations affect amino acids being important for proper folding and dimerization of the hormone especially corresponding to neurophysin II domain of the AVP prohormone. Neurophysin II (and neurophysin I) contain a single hormone-binding site for which oxytocin and AVP do have identical affinity and binding kinetics. Neurophysin can self-associate by aggregation and dimerize and hormone binding is indeed the main factor involved in disturbing these processes. There are experimental evidences which have shown that heterologous expression of disease-associated *AVP* gene variations in cell cultures and transgenic animals may guide to the making of a variant AVP hormone precursor which unlike wild type is processed inefficiently into AVP and is thus retained in ER (Siggaard et al. 2005). On the other hand variant protein arising from recessive form of FNDI affects final processing after ER rather than the ER-mediated transport of the hormone precursor (Christensen et al. 2004a, b). It supports that dominant form of FNDI causes misfolding and aggregation of variant AVP in ER itself (i.e., misfolding neurotoxicity hypothesis).

6.2.2 Lethal Protein Misfolding Outcomes and Loss of Function

Protein misfolded diseases where proteins tend to form aggregates and lose their normal functioning lead to several lethal diseases.

6.2.2.1 Cystic Fibrosis

Cystic fibrosis (CF) is a deadly autosomal recessive disease in which there occurs a mutation in the cystic fibrosis transmembrane conductance regulator gene (CFTR), coding for the chloride ion channel of membrane of epithelial cells, particularly of pulmonary tissues (Bobadilla et al. 2002). Because of this impairment, it has been found that both intestines and lungs have thick mucous secretions present (Muchowski 2002). It has been reported that about 1,000 disease-associated CFTR *g*ene mutations are possible. Among them, the most common one in which phenylalanine at position 508 in the primary structure is deleted and is denoted as ΔF508. About 66 % of all diseases of CFTR mutation are associated with this mutation (Bobadilla et al.

2002). Because of this mutation, CFTR protein is not trafficked out of ER and as a result its maturation gets blocked which leads to its premature proteolysis. This mutation accounts for 3/4 of CF cases (James et al. 1999). Cheng and co-workers (1990) made the important observation that the ΔF508 mutant CFTR did not attain the mature glycosylation state because it was retained in the ER and could not reach Golgi apparatus where from it could have been expressed outside the cell through plasma membrane. The trafficking defect has been found to be quite evident in tissues which have been taken from CF patients (Kartner et al. 1992)

Studies on a set of chemically synthesized polypeptides demonstrated that the physical basis of the ΔF508 mutation was again misfolding and destabilization of the polypeptide conformation and this indicates that this form of cystic fibrosis is the category of protein misfolding disease (Thomas et al. 1992a, b). This work led to hypothesis that conditions which would stabilize the crucial conformer may reverse the disease phenotype (Thomas et al. 1992a) – a prediction that was subsequently borne out by the finding that at low growth temperatures, the maturation efficiency of Δ F508 CFTR is increased (Denning et al. 1992) . The benefit of correcting the folding/maturation defect for the CF patient is clear from the demonstration that ΔF508 CFTR somehow could attain native structure back by retaining at least some ability to function properly (Thomas et al. 1992b; Denning et al. 1992).

6.2.2.2 Alpha-1-Antitrypsin Deficiency

In Alpha-1-antitrypsin deficiency, glutamate at position 342 of α1-antitrypsin (α_1AT) gene is mutated by lysine. Like all other situations, if such a protein will misfold it will lead to polymerization and then subsequent aggregation inside the cells. Such an aggregate will not be released from cells and thus will be deficient in the blood. α_1AT protein belongs to family of serpins with serine at its active site and is an inhibitor of proteolytic enzymes. It has been found that it undergoes conformational change on binding to its target proteins. Synthesis of α_1AT takes place in liver and is then secreted in the blood plasma. It basically protects the tissues for neutrophil elastases which can lead to proteolysis. A lot of tissue damage and bleeding disorders can happen if there occurs imbalance between proteases and their serpin inhibitors. A number of defective α_1AT genetic variants have been reported till date. Misfolding of α_1AT can lead to its aggregation in hepatocyte ER, the site of its synthesis. These aggregated products accumulate and cause liver cirrhosis and subsequent α_1AT deficit in plasma which becomes a reason for pulmonary emphysema. The defective α1AT proteins lead to good degree of its polymerization which in turn causes hepatic inclusions and additional plasma deficiency of α_1AT.

Because of its substantial genetic variability, almost 90 naturally occurring variants (Pi types) of α_1AT have been observed. Almost 1 in 1,800 newborns is affected with severe α_1AT deficiency and 95 % of such individuals are homozygous for the PiZ allele (E342K). Homozygotes for E342K have lack of α_1AT as a result of which the lungs cannot protect the extensive damage of connective tissue proteins by elastases of neutrophils. These individuals develop premature pulmonary emphysema in adult life (Lomas and Parfrey 2004). The E342K substitution decreases stability of monomeric forms of α_1AT leading to polymerization by the 'loop-sheet' insertion mechanism. This PiZ α_1AT variant now forms aggregates in ER and is thus not released into circulation. A subset of patients who are born with homozygous PiZ α_1AT deficiency during their childhood develop chronic liver disease. In this situation toxic gain-of-function mechanism comes into play in which the accumulation and retention of the mutated protein causes cell damage by triggering some specific signal transduction pathways for proinflammation, apoptosis and autophagy (Teckman et al. 2004).

6.2.2.3 p53 Inactivation

p53, a known tumor suppressor protein, is a sequence-specific transcription factor and has important role in maintaining genome integrity. Almost every second human carcinoma has inactivated p53 and here again protein misfolding is the cause. p53 controls uncontrolled cell growth

and proliferation. Usually p53 is present at low concentrations in the cell; however, increased levels are detected when DNA damage is sensed to start protective measures. p53 being a transcription factor binds to a number of regulatory sites in the DNA which in turn leads to synthesis of proteins that are crucial to regulate cell division and proliferation by binding. However, it has been found that mutation frequency of p53 suppressor gene is very high in various human cancers (Teckman et al. 2004). These mutations are mostly missense and affect structure of this tumor suppressor gene and as result of the misfolding it is rendered non-functional. In this scenario defective p53 is not able to protect cells from unregulated and uncontrolled growth and thus paves way for cancerous growth and its progression.

Under normal circumstances, p53 is rapidly degraded by ubiquitin proteosomal system dependent on the ubiquitin ligase, MDM2. DNA damage leads to p53 phosphorylation which dissociates p53 from MDM2 and thus p53 is not ubiquitinated and so not degraded. DNA damage on the other hand leads to more MDM2 degradation and so p53 is further preserved. At times of genetic stress DNA damage leads to preservation of p53 which in turn leads to transcription of genes involved in cell growth, division and its regulation. The oncogenic mutations that happen in p53 alter its core structure and do not allow this protein to attain native functional structural form. The mutations in p53 affect it in several ways. Firstly the mutant p53 forms although interact with other p53 proteins and forms tetramers but these are not functional. Second mutant p53 is not degraded as it does not interact with MDM2. Furthermore, molecular chaperones like HSP90 can stabilize the mutant p53. Because of this, formation of a wild-type functional tetramer becomes all the more impossible.

A large number of strategies have been developed to combat p53 mutations. One such step is use of small molecules called nutlins. Nutlin does not allow MDM2 to interact and bind with wild-type p53 and thus prevent its degradation. Another strategy is to design small molecules that can interact with mutant p53 and restore their functionality (Gavrin et al. 2012). One such compound pk7088 can stabilize Y220C p53 mutant and can revert its transcriptional activity to that of the wild-type protein (Liu et al. 2013). Y220C is mutated in large number of human cancers and so this brings up the hope that once the mutation is identified a person can be treated at individual level for a particular cancer.

6.2.2.4 Fabry's Disease

Fabry's disease, a lysosomal storage disorder, is again a protein conformational disorder. It presents a deficiency of lysosomal galactosidase A activity which in turn leads to the accumulation of glycosphingolipid globotriosylceramide (Gb3). In most of the cardiac Fabry patients, missense mutations have been found in the α-Gal A gene. In addition, minor deletions and alternative splicing mutations are also reported (Ishii et al. 2002). Mutations in these enzymes do lead to their misfolding. These misfolded enzymes in turn are taken up by the protein quality control system of ER and are therefore degraded before being sorted to lysosome. It has been elucidated out that missense mutations that lead to misfolding of α-Gal A do certainly lead to Fabry's disease.

6.2.2.5 Phenylketonuria

Phenylketonuria (PKU) is an inborn metabolic error disorder in which mutation takes place in the phenylalanine hydroxylase (PAH) gene that in turn makes the protein prone for enhanced degradation. Structurally, the functional form of the enzyme is homotetrameric in nature and each of its homo subunit has been found to be made up of three functional domains – the regulatory domain towards N-terminal side (residues 1–142), the catalytic domain (residues 143–410), including sites for substrate and co-factor binding; and the C-terminal oligomerization domain (residues 411–452). Any changes in the primary sequence of PAH protein causes its misfolding and in turn prevents its active tetramer formation leading to its enhanced degradation. As a result there is no functional enzyme present in the cell and so PKU represents loss-of-function pathogenesis. Waters and his group (2003) have shown that at molecular level protein misfolding is the leading cause of PAH by showing that the variant PAH protein undergoes defective oligomerization, reduced stability and enhanced degradation.

6.2.2.6 Sickle-Cell Anemia

Sickle-cell anemia is a genetic disorder in which valine at sixth position of the β-globin chain is mutated by glutamine. In sickle-cell anemia, haemoglobin (Hb) is mutated and forms stable long polymer fibres due to extensive binding between adjacent haemoglobin chains. This polymerization happens also when mutant haemoglobin S (HbS) is in deoxy state. RBCs change in shape and rigidity owing to this polymerization. These β-pleated sheet structures accumulate as 'amyloid plaques'. Detectable haematologic signs of the disease can be easily seen after 10 weeks. At the age of 6–12 months, symptoms of the diseases are quite clear with a decreased concentration of circulating foetal haemoglobin which under normal conditions protects aggregation of haemoglobin S (HbS). Interestingly, it has been shown that above its critical solubility limit, deoxygenated HbS polymerizes into fibres. Polymerization of Hbs into rigid fibres does in turn distort the shape of the erythrocytes. The activation of polymerization is largely influenced by molecular crowding. Polymerization involves a nucleation step and the addition of crowding agents has been found to increase the growth rate of the polymer by 10^4–10^5 fold (Ferrone and Rotter 2004). Thus this aggregated form of haemoglobin severely affects the normal physiological functioning of red blood cells and causes severe haematological disorders. The Gain of (Toxic) function diseases and Loss of Function diseases are summarised in (Table 6.1) as given below:

Table 6.1 List of folding disorders in categories of gain of (toxic) function and loss of function

Gain of (toxic) function transformations	Loss of function transformations
Alzheimer's disease	Cystic fibrosis
Parkinson's disease	Alpah-1-antitrypsin deficiency
Transmissible spongiform encephalopathy	P53 inactivation
Huntington's disease	Fabry's disease
Amyloid lateral sclerosis	Sickle cell anemia
Familial neurohypophyseal diabetes insipidous	Phenyl ketonuria

6.3 Conclusion and Future Perspective

Misfolding of proteins and defects in their processing and degradation pathways have been found to be the most common features of neurodegenerative diseases. Because of this, misfolded structures are built up in and out of the cell. Understanding proteopathies and then developing suitable therapy is still a challenge for the scientists working in this area, even though a number of genetic variations have been identified till now in case of protein misfolding diseases especially in neurodegenerative diseases. Protein misfolding as has been discussed earlier can have various consequences – firstly toxic fibrils may develop and lead to cell death, second these misfolded proteins target the various proteasome degradation pathways and so they become deficient in the cells and lastly these β-structure-rich misfolded structures associate with each other and form large macromolecular structures which get deposited inside cells and tissues and lead to their degeneration. Therefore such strategies are being worked out whereby these β sheet structures can be reversed and the protein persuaded to get folded into native conformation.

This will not only pave a way out for tackling these awful diseases but will also give relief to millions whose life changes for ever by these inane mutations which have somehow evolved during time and are making proteins go astray. Further down the line this will revolutionize pharmacodynamic strategy of designing drugs that have the ability to reverse protein misfolding in specific targets and will open new avenues for giants in pharmacy business to tap the resulting market owing to wide incidence of such diseases and will further strengthen the concept of personalized medication and treatment. Protein misfolding leads to a number of diseases, collectively called as proteopathies. So a specific protein gets misfolded and never achieves its native conformation whereby it will either form aggregates which are associated with proteasome degradation pathways and become deficient in terms of its normal functional levels in the cells so referred to

as 'loss of function' pathogenesis or in other case the misfolded protein forms toxic amyloid structures/fibrils which act as infectious agents and lead to misfolding of other proteins, this is referred to as 'toxic gain of function' pathogenesis (Valastyan and Lindquist 2014). These observations suggest that the molecular origin of such protein misfolding diseases may ultimately be traced back to the presence, in cells, of protein or peptide molecules with incorrect or abnormal structures, different from those which are normally found in functional living cells (Stefani and Rigacci 2013). Proteins such as Aβ, tau, prion protein and α-SYN which have amyloidogenic tendencies are accumulated in various neurodegenerative diseases and achieve a toxic gain of function. Current investigations have shown that toxic effects are mediated by soluble oligomeric assemblies and not by amyloid fibrils. In case of other neurodegenerative diseases like PD, CF, αAT, FD, etc., the majority is involved in pathogenesis through a loss of function. In such diseases, deficit of a neuroprotective factor is related to impaired neuronal vulnerability. Literature studies have shown that PrP^c and α-SYN can have a neuroprotective capability, which is no longer possible in their misfolded states. Based on our understanding of ND, can we devise a therapeutic strategy for these NDs is an important question. It can be seen that all protein misfolding diseases share common features, which suggest a common harmful potential of these diseases. Literature studies have shown that anti-Aβ vaccination can prevent cognitive deficits in mouse models of AD. Besides this, modulation of PQC machinery may serve as an interesting therapeutic strategy which acts as a first-line defensive system to prevent misfolding.

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Protein Misfolding and Amyloid Formation in Alzheimer's Disease

7

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Contents

Abstract

The information necessary for proteins to correctly fold into biologically active three dimensional (3D) structures is present in the amino acid sequence. The ways by which proteins fold still remain one of the unexplained mysteries in the field of protein biochemistry. Investigating the impact and consequences of protein misfolding can help decipher the molecular causes behind the complex amyloid diseases such as Alzheimer's disease (AD) and Parkinson's disease. Various participating molecular entities like amyloid beta (Aβ), tau protein, and non-beta sheets are facilitating the pathogenesis of Alzheimer's disease. Understanding their structure as well as their mechanism of action is useful to decode the therapeutic treatment for these complex diseases.

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7.1 Introduction

One of the mystifying paradigms in biochemistry is to understand the dilemma of protein folding. In recent years, with the discovery of numerous diseases as protein folding disorders and with the sudden increase in the genomic information and the requirement for proficient methods to predict protein structure, protein folding developed into a fundamental subject in biological sciences (Brito et al. 2004). Protein folding diseases can be classified into two sets: (1) in first, extreme amounts of incorrectly folded proteins accumulate as unfolded proteins. This group represents amyloidosis diseases, such as Alzheimer's disease (2) in this group, a little fault in the genetic outline directs towards partial folding of a protein, thereby affecting its function.

Protein folding is accepted as a crucial issue in the twenty-first century. To correctly predict protein-folding patterns is tricky owing to the complex structure of proteins (Chen et al. 2012). In order to carry out their biological functions, all proteins should fold into specific three-dimensional structures. However, the genetic information for the protein gives merely the linear chain of amino acid residues (the primary structure) in the polypeptide backbone. The dilemma of protein folding can be broken down into three different nevertheless correlated issues: (1) kinetic process or pathway responsible for the native and biologically active conformation of the proteins; (2) the physical basis accountable for the stability of folded conformations; (3) determination of one particular folding process by the amino acid sequence, and resulting three-dimensional structure (Creighton 1990).

7.2 Protein Misfolding

Folding and unfolding are the crucial ways of producing and eliminating explicit forms of cellular activity. Also, processes such as translocation across membranes, trafficking, secretion, the immune response and regulation of the cell cycle are directly dependent on folding and unfolding proceedings (Radford and Dobson 1999). There are different levels of structures into which the protein chains can fold, however, a small number of fundamental confined folds, or secondary structures (for example; helices and beta sheets), are prevalent. Folding *in vivo* is a co-translational event with few an occasions, which means that it commences way ahead before the completion of protein synthesis with the growing chain still attached to the ribosome (Hardesty and Kramer 2001). For some proteins, on the other hand, the major part of their folding undergoes in the cytoplasm following their release from the ribosome. However, there are others that fold in specialized organelles, for example mitochondria or the endoplasmic reticulum (ER), after trafficking and translocation all the way through membranes (Bukau and Horwich 1998).

Specific proteins particularly molecular chaperones are, consequently, essential to aid proteins in folding and to avoid aggregation of folding intermediates. Failure to fold precisely, or to stay accurately folded, will as a result confer increase in the malfunctioning of living systems and consequently to disease (Horwich 2002). Several of these diseases, for instance, cystic fibrosis (Thomas et al. 1995) and a few types of cancer (Bullock and Fersht 2001), result from incorrect folding of proteins and not being capable to put into effect their appropriate function. Many of these disorders are familial since the possibility of misfolding is frequently larger in mutational variants. In cases of proteins that have an elevated inclination to misfold, escape the entire protective machinery and form unmanageable aggregates inside the cells or in extracellular space. A growing amount of disorders, together with Alzheimer's and Parkinson's diseases, the spongiform encephalopathies and type II diabetes, are directly related with the deposition of such aggre-

gates in tissues, such as brain, heart and spleen (Horwich 2002; Dobson 2003; Aguzzi and Calella 2009).

Protein folding diseases can be classified into two sets:

1. Firstly, as unfolded proteins, which are the result of abnormal accumulation of incorrectly folded proteins. The proteins in this group majorly are prevalent in causing amyloidosis diseases, such as Alzheimer's disease.
2. Secondly, due to minor faults in the genetic outline that directs towards partial folding of a protein, thereby affecting its function.

One of the simplest is to unfold the protein in a high concentration of chemical denaturant, such as guanidium chloride, and then dilute the solution rapidly, such that the denaturant concentration falls below that at which the native state is thermodynamically unstable. Another strategy that has been developed is to use a battery of complementary stopped-flow and quenched-flow techniques, each of which is capable of monitoring a specific aspect of the formation of native-like structure (Dobson et al. 1994). In addition, novel methods to initiate refolding reactions are being introduced (Ballew et al. 1996). These methods include the use of temperature jumps under conditions where cold denaturation takes place and an increase in temperature leads to refolding. In some cases, the rapid change of oxidation state of a metalloprotein can trigger the onset of the folding reaction (Pascher et al. 1996). With such approaches, folding events on the micro- and sub-micro-second time scale is becoming accessible (Dobson et al. 1998).

7.3 Amyloid Formation of Proteins

Protein misfolding leads to malfunctioning of cellular machinery, hence consequently resulting in a wide spectrum of diseases. One of these events being amyloid, where protein fragments or whole protein unfolds from their soluble forms to insoluble fibrils. Most of the times, these fibrils appear in the form of β-sheet, hence the term β-amyloid. These fibrils accumulate in different organs and lead to corresponding different diseases.

The basic amyloid structure contrasts strongly with the extremely original globular structures of most natural proteins. In these latter structures, the interactions linked with the exceedingly definite packing of the side chains appear to make ineffective the main-chain preferences (Dobson 1999). The polypeptide main chain and the hydrophobic side chains of a globular protein are basically concealed inside the folded structure. The switch into amyloid fibrils will be feasible only when they are in an exposed state, such as once the protein is partially unfolded (at low pH) or fragmented (by proteolysis). *In vitro* experiments show that their formation is then usually characterized by a lag phase, followed by a phase of quick growth (Caughey and Lansbury 2003). This behavior is characteristic of nucleated processes for instance crystallization; the lag phase can be removed by adding up of preformed aggregates to new solutions, a method well-known as seeding. An appealing proposal is that seeding by chemically tailored forms of proteins, resulting from deamidation or oxidative stress, might in various cases be a vital aspect in activating the aggregation process and the inception of disease (Nilsson et al. 2002). The aggregates that are formed initially are expected to be comparatively disorganized structures that expose to the outer environment a diversity of parts of the protein that are in general buried in the globular state (Bucciantini et al. 2002; Dobson 2003).

Amyloid fibrils represent just one of the kinds of aggregates which are produced by proteins, even though a considerable attribute of this particular group is that its extremely ordered hydrogen-bonded structure is expected to bestow its exceptional kinetic steadiness. Therefore, once formed, these aggregates can persevere for extensive times, permitting a progressive increase of deposits in tissue, and in fact facilitating seeding of the successive translation of additional amounts of the same protein into amyloid fibrils.

All amyloid deposits demonstrate definite optical behavior (such as birefringence) on binding to specific dyes such as Congo red. The latter discloses that the ordered core arrangement is composed of β-sheets whose strands run perpendicular to the fibril axis (Sunde and Blake 1997). The most persuasive confirmation for the last proclamation is that fibrils can be produced *in vitro* by numerous other peptides and proteins, together with distinguished molecules for instance, myoglobin, as well as by homopolymers such as polythreonine or polylysine (Dobson 2001, 2003; Yoon and Welsh 2004; Xu et al. 2010).

The central structure of the fibrils appears to be steadied mainly by interactions, predominantly hydrogen bonds, concerning the polypeptide main chain. Since the main chain is universal to all polypeptides, this observation describes why fibrils produced from polypeptides of extremely dissimilar sequence look like to be so similar (Dobson 1999). In a few cases, merely a handful of the residues of a particular protein might be implicated in this structure, with the rest of the chain being coupled in some other way with the fibrillar assembly; in others approximately the entire polypeptide chain seems to be occupied. Regardless of these complications, in the past few years, various experimental methods have come into view that allows the recognition and the thorough molecular investigation of misfolded oligomers (Bemporad and Chiti 2012).

In addition, the comparative aggregation rates for an extensive collection of peptides and proteins correlates with the physicochemical characteristics of the molecules for example charge, secondary-structure propensities and hydrophobicity (Chiti et al. 2003). Further, in numerous occasions, surface charges of the proteins have been established to function as "structural gatekeepers," that shun unnecessary interactions by negative design, such as, in the control of protein aggregation and binding (Kurnik et al. 2012).

Once a protein turns into toxic, a widespread conformational transformation takes place and it attains a motif, for instance the β-sheet. It may be noted that the β-sheet conformation is present in several functional native proteins like immunoglobulins; however, the conversion from α-helix to β-sheet is a feature of amyloid deposits (Kirkitadze et al. 2001). The uncharacteristic conformational change from α-helix to β-sheet exposes hydrophobic amino acids and encourages protein aggregation. The toxic arrangement is frequently capable of interacting with other native proteins and catalyzes their conversion into the toxic condition. Because of this capacity, they are known as infective conformations. The freshly made toxic proteins replicate the rotation in a self-sustaining loop, augmenting the toxicity and consequently leading to a disastrous result that finally destroys the cell or harms its function (Reynaud 2010).

Proteins carry out their normal functions accurately when the chains of amino acids, from which they are built, fold perfectly. Misfolded proteins can be toxic to the cells and amass into insoluble aggregates with other proteins. Ataxin-1 is extremely prone to misfolding owing to inherited flaws in the gene that lead to neurodegenerative diseases, as the glutamine amino acid is recurring in the protein chain of ataxin-1. Higher the number of glutamine residues, the greater toxic is the protein (La Spada and Taylor 2010). There are about 21 proteins that generally interact with ataxin-1 and manipulates its folding or misfolding such as GTP-binding nuclear protein Ran and adapter molecule Crk protein. Studies suggest 12 of these proteins increase the toxicity of ataxin-1 for the nerve cells, while 9 of the identified proteins reduce its toxicity (Petrakis et al. 2012). This structure endorses aggregation, for the reason that the proteins that interact with ataxin-1 and possess this domain increase the toxicity of mutated ataxin-1 (Tsuda et al. 2005).

7.4 Protein Folding Diseases with Respect to Amyloid Formation

Accumulation of misfolded proteins can cause amyloid disease; the most prevalent one is Alzheimer's disease, while Parkinson's and Huntington's disease have related amyloid origins (Haass and Selkoe 2007). Every amyloid

disease involves primarily the aggregation of an explicit protein, even though a variety of other machinery together with supplementary proteins and carbohydrates are included in the deposits as soon as they are formed *in vivo*. Amyloid accumulation occurs in different parts of the body, leading to malfunctioning of bodily processes, which is termed as amyloid disease. The huge amounts of insoluble protein involved in various aggregation diseases can physically disturb particular organs and in doing so cause pathological behavior (Tan and Pepys 1994). Based on frequency of occurrence, amyloid diseases can be classified as sporadic or familial.

For neurodegenerative disorders, like Alzheimer's disease, the principal indications more or less definitely are a consequence from a 'toxic gain of function' related to aggregation (Taylor et al. 2002; Dobson 2003). Aggregation of the microtubule coupled protein tau is linked with numerous neurodegenerative disorders, together with Alzheimer's disease and front temporal dementia (Kfoury et al. 2012). In addition, the malfunction of proteins to fold accurately and efficiently is being linked with the failure of biological systems, and considerable varieties of diseases are currently identified to be related with the misfolding of proteins. A few of these diseases, for instance cystic fibrosis, result from mutations, which obstructs with the standard folding and secretion of particular proteins. Others, like Alzheimers and Creutzfeldt-Jakob diseases, are linked to the later switch of typical soluble proteins into insoluble amyloid plaques and fibrils (Prusiner 1992). Population of helical intermediates and their stabilization by means of interactions with membranes may be a significant cause by which the progression of aggregation directs to toxicity (Pappu and Nussinov 2009).

The danger of achieving any of these diseases amplifies considerably with age. The mechanistic justification for this relationship is that with age (or due to mutations), the subtle equilibrium of the synthesis, folding, and degradation of proteins is perturbed, following in the production and increase of misfolded proteins that form aggregates (Lindholm et al. 2006).

Amongst the environmental factors which raise the possibility of suffering degenerative diseases is contact to substances that have an effect on the mitochondria, resulting in enhancement of oxidative harm to proteins (Jenner and Olanow 1996). On the other hand, it is apparent that no single environmental factor is accountable. Besides, there are genetic factors as well. For instance, in the simplest forms of familial Parkinson's disease, mutations are related with dominant forms of the disease. In Alzheimer's disease, and for other fewer widespread neurodegenerative diseases, the genetics can be still extra difficult, because diverse mutations of the similar gene and combinations of these mutations may influence disease risk in a different way (Bertram and Tanzi 2004).

7.5 Alzeihmer's Disease as a Protein Folding Disease

7.5.1 Mechanism

The causal pathway of Alzheimer's disease (AD) is a complex mechanism comprising of different proteins. As per amyloid cascade hypothesis, AD is caused due to the formation of a peptide (protein) known as amyloid beta (or beta amyloid, Aβ), which clusters as senile plaques on the blood vessels and accumulation of amyloid fibrils on the outside surface of neurons of the brain leading to the destruction of neurons. This in turn inhibits the conventional impulses being transmitted by the affected neurons. Aβ has been shown to impair mitochondrial function in PC12 cells (Pereira et al. 1998). Aβ peptide is created by enzyme clipping of the normal neuron membrane protein known as amyloid precursor protein (APP) (Hartmann et al. 1997). Enzymes can clip APP in ways that do not result in Aβ formation. Moreover, there are two forms of amyloid beta peptide, one of which has 40 amino acids and another one that has 42 amino acids. The enzymes that cleave APP are known as secretases (Lathia et al. 2008). The two enzymes that initially compete to cleave APP are alpha-secretase (α-secretase) and beta-secretase (β-secretase,

BACE1) (Lathia et al. 2008). If alpha-secretase cleaves APP, there is no formation of Aβ. If APP is cleaved by beta-secretase it can then be further cleaved by gamma-secretase (γ-secretase) to form either a 40 amino acid amyloid peptide ($Aβ_{40}$) which is soluble and mostly innocuous—or a 42 amino acid peptide ($Aβ_{42}$) which clumps together to form insoluble amyloid plaques (Hartmann et al. 1997). Alpha-secretase cleavage occurs at the cell surface, whereas beta-secretase acts at the endoplasmic reticulum (Hartmann et al. 1997). Gamma-secretase produces $Aβ_{42}$ if cleavage occurs in the endoplasmic reticulum and $Aβ_{40}$ if the cleavage is in the trans-Golgi network (Hartmann et al. 1997).

7.5.2 Aβ Peptide Oligomerization

Aβ can exist as various aggregation states such as monomers, oligomers and eventually insoluble fibrils. The broad term 'oligomers' comprises diverse types of assembly for instance dimers, trimers, protofibrils, ADDLs (Aβ-derived diffusible ligands) and annular or pore-like oligomers. It was also recommended that oligomers may possibly be categorized into prefibrillar or fibrillar oligomers as they have dissimilar aggregation pathways (Cerf et al. 2009; Bitan et al. 2005).

Aβ oligomers are tiny, soluble oligomers, which consist of five or six monomer components or ADDLs and protofibrils (Baumketner et al. 2006; Oda et al. 1995; Younkin 1995; Lambert et al. 1998; Wang et al. 1999; Klein et al. 2001). The deposition of Aβ is slow and prolonged and may possibly extend for over two decades (Villemagne et al. 2013). The process of amyloid plaque formation starts with an increased production of total Aβ and Aβ42/Aβ40 ratio, which gradually leads into oligomerization of $Aβ_{42}$ and ultimately forms $Aβ_{42}$ deposits that result in inflammatory responses, astrocytic activation, synaptic spine loss and neuritic dystrophy (Haass and Selkoe 2007). It has been also reported in the literature that the structure of Aβ rather than sequence plays the principle role in Aβ induced toxicity (Celej et al. 2012). β-Sheets have been reported as dominating structures in Aβ oligomers (Cerf et al. 2009). The studies have also reported spectral similarities between Aβ oligomers and pore-forming porins and suggested that the ability of Aβ oligomers to form a porin-like structure might be associated with their toxicity in AD. Currently, inhibiting aggregation of the Aβ peptide by rational design of small inhibitor molecules may be a daunting task because of the unavailability of the high-resolution structure of toxic Aβ aggregate. However, other approaches such as high-throughput screening successfully identified certain compounds exhibiting promising inhibitory effect on Aβ aggregation (McKoy et al. 2014).

Process of Aβ fibrillation involves a conformational shift, which ultimately leads to the formation of extended β-sheets (Kirkitadze et al. 2001). Involvement of an oligomeric α-helix containing intermediate was also proposed as a key step in Aβ fibrillogenesis (Kirkitadze et al. 2001). Aβ peptide 1–40 fibril polymorphs share a common parallel β-sheet organization and possess similar peptide conformations, but differ in overall symmetry and in other structural aspects. In one of the study on disease-associated mutant, D23N $Aβ_{1–40}$, researchers have reported stabilized parallel and antiparallel β-sheets within amyloid fibrils (Sawaya et al. 2007). The role of antiparallel β-sheet structures are suggested to fibrils that are formed by short peptides with one β-strand segment only.

Certain structural models for Aβ fibrils contain a β-hairpin with intramolecular backbone hydrogen bonding between β-strand segments on either side of a β-turn present between Val-24 and Asn27 (Lazo and Downing 1998), Gly-25 and Lys-28 (Balbach et al. 2002; Li et al. 1999; George and Howlett 1999), or Ile32 and Gly-37 (Tjernberg et al. 1999). A variety of other aggregates have also been identified, including Aβ protofibrils and soluble oligomers of various sizes. NMR (nuclear magnetic resonance) studies suggest (Bertini et al. 2011; Lansbury et al. 1995; Paravastu et al. 2008; Petkova et al. 2005, 2006) Aβ fibrils are highly polymorphic, with molecular structures that depend on aggregation conditions. Detailed structural models for fibrils formed in vitro have been developed from experimental data showing that fibril polymorphs can

differ in specific aspects of peptide conformation and inter-residue interactions as well as overall structural symmetry (Bertini et al. 2011; Lührs et al. 2005). Aβ aggregates, also formed *in vitro*, provide evidence for peptide conformations similar to those in fibrils but with reduced structural order and different supramolecular organizations. Molecular structures of Aβ aggregates that develop in human brain tissue have not been characterized in detail, but evidence exists that structural variations may be biomedically important: (1) structurally distinct fibrils can have different levels of toxicity in neuronal cell cultures (Petkova et al. 2005); (2) propagation of exogenous Aβ amyloid within transgenic mouse brains depends on the source of the exogenous Aβ-containing material (Meyer-Luehmann et al. 2006; Stöhr et al. 2012). Experiments on tissue from two Alzheimer's disease patients with distinct clinical histories showed a single predominant 40 residue Aβ ($A\beta_{40}$) fibril structure in each patient; however, the structures were different from one another. A molecular structural model developed for $A\beta_{40}$ fibrils from one patient reveals features that distinguish *in vivo* from *in vitro* produced fibrils. The data suggest that fibrils in the brain may spread from a single nucleation site, that structural variations may correlate with variations in AD and that structure-specific amyloid imaging agents may be an important future goal (Lu et al. 2013).

Numerous models of the Aβ fold in amyloid fibrils have been anticipated, occasionally merging structural constraints of diverse experimental techniques or from samples that form under dissimilar conditions (Fändrich et al. 2011). Nevertheless, different circumstances can generate fibrils with unlike peptide conformation. Generally fibril models consider a U-shaped peptide fold, which is termed a β-arc (or β-arch) (Fändrich et al. 2011). U-shaped peptide models are derived from molecular dynamics simulations, partly applying structural limitations from solid-state NMR, or other biophysical methods. At times, it has been recommended that these models fit cryo-EM reconstructions (Fändrich et al. 2011). On the other hand, reconstruction of an $A\beta_{1–40}$ fibril at elevated resolution (0.8 nm), in which the cross β-sheet structure is directly determined, does not conform to the previous U-shaped peptide models (Fändrich et al. 2011). The 0.8 nm reconstruction presents cross-sectional dimensions that are significantly outsized than those predicted by the U-shaped models, and hence, ought to cover the peptide in a different structural arrangement (Fändrich et al. 2011).

7.5.3 Role of Aβ Structure in Alzheimer's Disease

In general, amyloids are large elongated structures having varying lengths and ultrastructural forms that make NMR and X-ray crystallography difficult (Toyama and Weissman 2011). Prion Het-s from fungus *Podospora anserine*, peptide Aβ related to AD and X-ray structures of short amyloidogenic peptides from various amyloid systems are a few of the well-studied amyloids. The most important structural feature of amyloid fibers is primarily a β-sheet structure, either in parallel and antiparallel form (Toyama and Weissman 2011) (Fig. 7.1). In amyloid structures, β-sheets can come together into a tertiary fold in a variety of different ways such as β-sandwich (Prion Het-s) or β-solinoid (Aβ) (Toyama and Weissman 2011).

In general, two types of aggregates are found in the brains of AD patients (Toyama and Weissman 2011) viz., intracellular neurofibrillary tangles (aggregates composed of the hyperphosphorylated protein tau) and extracellular plaques (made up of aggregates of the Aβ peptide)

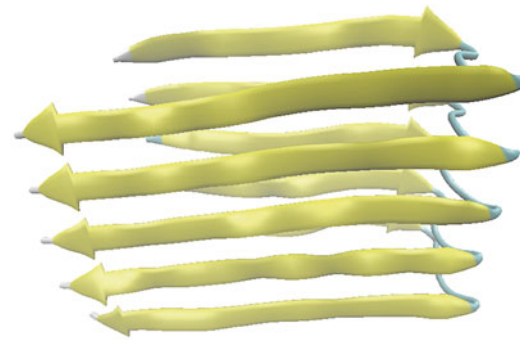

Fig. 7.1 3D structure of Alzheimer's Abeta (1–42) fibrils (PDB ID: 2BEG)

(Glenner and Wong 1984; Kosik et al. 1986). Aβ is a 39–42-residue peptide that originates from amyloid-β precursor protein (membrane protein) after proteolytic cleavage by β- and γ-secretases (Kang et al. 1987). X-ray fiber diffraction has previously revealed the presence of frequent cross-β diffraction pattern among extracellular plaques isolated from AD patients (Kirschner et al. 1986). Fourier Transform Infrared Spectroscopy (FTIR) and CD showed that the truncated Aβ peptide aggregates are mainly composed of β-sheet structures with β-sheets in an antiparallel arrangement (Lansbury et al. 1995). SSNMR has also played a significant role in understanding the high-resolution structures of Aβ fibers (Toyama and Weissman 2011). Early studies on truncated Aβ peptides ($A\beta_{34–42}$, $A\beta_{16–22}$, $A\beta_{11–25}$, and $A\beta_{14–23}$) also suggested antiparallel β-sheet structure (Lansbury et al. 1995; Balbach et al. 2000; Gordon et al. 2004; Bu et al. 2007). However, an in-register parallel sheet arrangement was observed for truncated $A\beta_{10–35}$ (Antzutkin et al. 2002). Interestingly, the SSNMR structures of full length $A\beta_{1–40}$ and $A\beta_{1–42}$ fibers revealed an in-register parallel β-sheet pattern (Antzutkin et al. 2000, 2002). From these studies it appeared that antiparallel β-sheet may perhaps be an artifact due to the truncation of hydrophobic and amphiphilic regions on Aβ (Toyama and Weissman 2011). Recently, a single predominant 40 residue Aβ ($A\beta_{40}$) fibril structure from two AD patients having different clinical histories and neuropathology was described by using simplified amyloid extraction and seeding methods (Lu et al. 2013).

The formation of amyloid fibrils can lead to a disease such as Alzheimer's disease, Diabetes type 2 and the spongiform encephalopathies (e.g., Mad cow disease), where each disease may be characterized by a particular protein or peptide that aggregates (Rambaran and Serpell 2008). In order to have a pathogenic consequence, these amyloid fibrils are deposited extracellularly in the tissues (Pepys 2001). However, all amyloid structures are not always cytotoxic as reported by the discovery of functional mammalian amyloid structure that functions in melanosome biogenesis (Fowler et al. 2006) that requires the formation of detergent-insoluble, lumenal Pmel17 fibers (Berson et al. 2003). Pmel17 is a transmembrane glycoprotein and its proteolytic processing yields a 28-kDa transmembrane fragment (Mβ) that degrades, and an 80-kDa lumenal fragment (Mα) which self-assembles into fibers that form the core of mature melanosomes (Marks and Seabra 2001; Berson et al. 2001, 2003). The recombinant Mα (rMα) accumulates more rapidly than any recognized polypeptide into amyloid fibers by at least four orders of magnitude quicker than the Aβ and α-synuclein peptides linked with Alzheimer and Parkinson disease (Fowler et al. 2006). Although some similarities have been observed between functional amyloidogenesis and pathogenic amyloid formation, they also exhibit remarkable differences. For instance, in case of gelsolin amyloid disease, proteolytic breakdown of mutant gelsolin during secretion by the proprotein convertase furin leads to slow, unregulated extracellular pathogenic gelsolin amyloid deposition (Chen et al. 2001). Similarly, Mα amyloid formation also begins by the activity of proprotein convertase but produces a functional amyloid structure (Fowler et al. 2006).

In addition to amyloid precursor protein, many other plasma proteins such as atrial natriuretic factor (ANF), wild-type transthyretin, β2-microglobulin, etc. have been identified that form amyloids (Fowler et al. 2006). Regardless of the noticeable differences in amino acid sequences and native structure of these amyloidogenic peptides, they all exhibit a widespread β-sheet conformation of their polypeptide backbone (Eanes and Glenner 1968; Sunde et al. 1997). Therefore, it is very much likely that this characteristic feature bestows the fibrillar, proteolytic resistant as well as insoluble characteristics to all types of amyloid (Fowler et al. 2006).

7.5.4 Conformational Changes in Aβ

Alteration in specific proteins or peptides from their native conformations and subsequent aggregation as insoluble fibrils has been proposed as causative agents in several neurodegenerative

disorders including AD (Celej et al. 2012). These conformational inequities in proteins explicate differences in various neural diseases (Guo et al. 2013). Growing evidence suggests abnormal accumulation of fibrillar Aβ in extraneuronal spaces and intraneuronal aggregates of abnormally modified tau proteins as a principal causation event in AD (Martha et al. 2012; Cerf et al. 2009). It is generally accepted that Aβ accumulation in brain is the primary event and tau protein formation could be one of the consequences of an imbalance between production and clearance of Aβ (Cerf et al. 2009). Up to 38–43 residues long Aβ released after the proteolytic cleavage of amyloid precursor protein have been reported as a primary component of amyloid plaques (Cerf et al. 2009)**.** The key components of these amyloid plaques are highly amyloidogenic, less abundant, early deposited $A\beta_{1–42}$ and $A\beta_{1–40}$ (Cerf et al. 2009).

There is evidence towards monomeric $A\beta_{42}$ peptides conformational states as building block of amyloid fibers (Baumketner et al. 2006). A conformational shift from α-helix to β-sheet of protein structure has been reported during the aggregation of amyloid fibrils associated with AD (Ding et al. 2003).

Although universal structure for Aβ fibrils does not exist, several antiparallel β-sheets structural models have been proposed by earlier studies (Chaney et al. 1998; George and Howlett 1999; Li et al. 1999; Tjernberg et al. 1999; Lazo and Downing 1998). Native Aβ peptide in AD is α-helix rich and polymorphic at molecular structural level (Tycko 2013; Ding et al. 2003).

Process of Aβ fibrillation involves a conformational shift which ultimately leads to the formation of extended β-sheets (Kirkitadze et al. 2001)**.** Involvement of an oligomeric α-helix containing intermediate was also proposed as a key step in Aβ fibrillogenesis (Kirkitadze et al. 2001). Aβ peptide 1–40 fibril polymorphs shares a common, parallel β-sheet organization and possess similar peptide conformations but differ in overall symmetry and in other structural aspects. In one of the study, on disease-associated mutant, D23N $A\beta_{1–40}$, researchers have reported stabilized parallel and antiparallel β-sheets within amyloid fibrils (Sawaya et al. 2007). The role of antiparallel β-sheet structures are suggested to fibrils that are formed by short peptides with one β-strand segment only.

Certain structural models for Aβ fibrils contain a β-hairpin with intramolecular backbone hydrogen bonding between β-strand segments on either side of a β-turn present between Val-24 and Asn27 (Lazo and Downing 1998), Gly-25 and Lys-28 (Balbach et al. 2000; Li et al. 1999; George and Howlett 1999), or Ile32 and Gly-37 (Tjernberg et al. 1999).

7.5.5 Misfolding in Neurofibril Tangles

The neurofilament is a linear 9–10 nm microfilament found in the neuronal cell body, the axon and the dendrites. It has an inadequately distinct lumen, short "side arms" project from it and it appears to be made up of globular subunits. Unlike neurotubules, the neurofilaments are steady and can be voluntarily secluded by subcellular fractionation. The neurofibrillary tangles (NFTs) are also found in the brain of normal aged humans (Iqbal et al. 1975). NFTs are aggregates of hyper phosphorylated tau protein that are generally identified as a main marker of Alzheimer's disease. Alzheimer's disease at the neuropathological stage is characterized by the build-up and aggregation of misfolded proteins: intracellular aggregates of tau in the NFTs and extracellular aggregates of Aβ deposits in forms of parenchymal amyloid plaques and congophilic amyloid angiopathy (CAA) (Hoozemans et al. 2005; Scholtzova et al. 2014).

The formation of amyloid plaques and NFTs are thought to contribute to the degradation of the neurons (nerve cells) in the brain and the subsequent symptoms of Alzheimer's disease (Strittmatter and Roses 1996). The conventional understanding is that tau attaches to microtubules and helps with their formation and stabilization. On the other hand, when tau is hyperphosphorylated, it is incapable to bind to microtubules and becomes unstable. The unbound tau clumps together in formations called NFTs (Spires-Jones

et al. 2009). These lesions, eventually, develop into filamentous NFTs, which hamper with several intracellular functions. Correlation among the quantitative aspects of Alzheimer's disease (neuron loss, neuritic plaque and neurofibrillary tangle load) and anger is normally observed in Alzheimer's patients. It was established that simply a raise in NFTs load was linked with the harshness of aggression and chronic aggression in Alzheimer's patients (Churchyard and Lees 1997).

Numerous interpretations of familial forms of Alzheimer's disease point towards that genetic factors resulting in alterations in β-amyloid are adequate to cause Alzheimer's disease, as well as tangles and other neurofibrillary changes (Hardy and Selkoe 2002). These observations offer strong confirmation that β-amyloid can generate or intensify neurofibrillary changes, at least following the early stages of amyloid deposition. Histologically, the neuronal cytoskeleton curls, factually, into structures known as NFTs. External to the cell, the Aβ peptide aggregates into clumps called oligomers, that gather and lead to the formation of amyloid plaques derived from the studies of a disorder identified as mild cognitive impairment (MCI) (a potential prodrome to dementia); the growth of noticeable entorhinal NFTs is measured to be the histologically correlate of MCI and, considered as the indication of early Alzheimer's disease (Gandy 2005).

7.5.6 Non-Beta Sheet Fibrils and Pathogenic Agents

General mechanism of aberrant protein aggregation has been reported by β-sheet conformation of Aβ fibrils. However, some studies also reported role of paired α- helical filaments (PHFs) conformation in the pathogenesis of AD (Sadqi et al. 2002). PHFs are abnormal twisted filaments, about 20 nm in width, and are composed of hyperphosphorylated tau protein (Ksiezak-Reding and Wall 2005). They are found in Alzheimer's disease, NFTs and other neurodegenerative disorders such as tauopathies (Braak and Braak 1991; Spillantini et al. 1997). PHFs have also been reported in brains of aging mammals (Cork et al. 1988; Schultz et al. 2000).

Moreover, tau-protein PHF's primary constituent has been also reported to contain high abundance of helix-breaking amino acids and is unstructured in solution (Sadqi et al. 2002). These internalized PHFs are capable of accelerating the formation of GFP-Tau-positive inclusions (aggresomes) in a pericentriolar location of the cells (Santa-Maria et al. 2012). It was also shown that PHFs from extracellular human AD can be internalized by cultured cells and may propagate a misfolded state to native soluble intracellular tau protein (Santa-Maria et al. 2012). PHFs enter the cells by an endocytic pathway known as fluid-phase endocytosis that involves engulfment by the cell membrane (Santa-Maria et al. 2012). However, additional studies are required to identify whether classical or rare endocytic pathways are responsible for internalization and cellular transport of PHFs (Hansen and Nichols 2009). Another mechanism for fibrillar polyglutamine aggregates (in Huntington disease) has been proposed, in which the aggregates were internalized by just crossing the membrane wall in various cell lines such as HEK 293 and N2a cells (Ren et al. 2009). The PHFs can be formed by the full-length human tau protein, as well as by the three or four repetitive tau segments (Yu et al. 2012).

Small oligomeric forms of α-synuclein have also been reported as a responsible factor of neuronal death (Conway et al. 2000). It has been reported that α-synuclein assemblies adopt a common cross-β structure with β-strands perpendicular to the fibril axis (Apetri et al. 2006). Both parallel and antiparallel β-sheet orientations have been reported in the amyloid fibrils (Apetri et al. 2006). In contrast, it was described that spheroidal α-synuclein oligomers are loaded with β-sheet structure and switch from monomer to oligomers involves a secondary structural shift from natively unfolded protein to primarily β-sheet (Volles et al. 2001; Apetri et al. 2006). Throughout the development of α-synuclein filaments, there is significant decrease in α-helical content and increases in β-sheet structure, whereas the

involvement of extended and PPII structure is negligible (Apetri et al. 2006). Higher toxicity of α-synuclein oligomers and their ability to destruct membranes has been reported both *in vitro* and *in vivo* models (Celej et al. 2012; Kim et al. 2009; van Rooijen et al. 2008; Kayed et al. 2004). Conformational organization of oligomeric α-synuclein contains β-sheet structural elements (Celej et al. 2012; van Rooijen et al. 2008). There is strong evidence which highlights multi-pathway aggregation of α-synuclein and must be considered for investigations concerned on molecular mechanisms of this protein fibrillation (Hong et al. 2011). Recently, it was shown that α-synuclein protein misfolding cyclic amplification (PMCA) technique may possibly be employed as a high throughput screening method for the discovery of new α-synuclein anti-aggregating compounds that may inhibit α-synuclein fibril formation (Herva et al. 2014). Some of the soluble Aβ oligomers are currently extensively accepted as main pathogenic structures in AD (Hefti et al. 2013).

Another insidious pathologic feature of AD called tauopathy has been also known to be promoted by oligomerization. The tau oligomers have been reported as a pathological structure associated with AD progression in mouse models and plays a key role in neurodegeneration and behavioral impairments (Davidowitz et al. 2008; Berger et al. 2007; Maeda et al. 2006). Higher toxicity of tau oligomers have been reported in scientific literatures compared with tau filaments and has been suggested as precursors of tau filaments (Davidowitz et al. 2008; Gómez-Ramos et al. 2006, 2008).

7.5.7 Role of Posttranslational Modifications in Alzheimer's Disease

The hyperphosphorylated varieties of microtubule associated protein (tau) have been proposed as another pathological hallmark of AD. Changes in the quantity or the structure of tau protein may possibly influence its function as a microtubules stabilizer (Kolarova et al. 2012). Several studies reported unusual posttranslational modifications like hyperphosphorylation, acetylation, glycation, nitration, truncation and others accountable for changed tau structure in AD (Kolarova et al. 2012; Mondragón-Rodríguez et al. 2009; Binder et al. 2005; Kuhla et al. 2007; Carrell and Gooptu 1998). Recently, it was anticipated that not just the amount of neurofibrillary tangles but also state of proteolysis at C-terminus (associated with conformational changes) defines AD progression (Kolarova et al. 2012). The studies on mutation and their adverse effects on the stability of the EPHB2 gene are inferred from the studies and its impact with its reactivity with NMDA receptor and the function in the AD (Tayubi et al. 2014).

7.5.8 Role of Metal Ions in Alzheimer's Disease

The bioavailable metal ions predominantly iron, zinc and copper play a crucial role in the pathogenesis of AD. The studies have provided a proof that normalizing metal ion represents a valid therapeutic target (Duce et al. 2011). Among accepted aggravating factors, metal ions like (Al, Zn, Cu and Fe) could precisely damage protein aggregation of Aβ; aggregation of Aβ by self-assembly into oligomers or amyloids is a central event in AD (Drago et al. 2008). Organization of metal ions, mainly copper and zinc, in vivo, which modulates the aggregation process in Aβ, and the impact of Cu and Zn on the aggregation of Aβ reveal some general trends (Faller et al. 2013). Some suggestion has been made about the Aβ precipitation, and toxicity in AD is caused by abnormal interactions with metal ions, especially Zn, Cu and Fe. However, Aβ might also participate in normal metal-ion homeostasis (Bush 2003). Mainly the concentrations of Cu and Zn affects the types of aggregation intermediates formed and the binding of metal ions changes both the structure and charge of Aβ in Alzheimer's disease (Faller et al. 2013). The decrease in overall charge at physiological pH increases overall

driving force for aggregation, but may favour more precipitation over fibrillation, whereas the induced structural changes seem more relevant for the amyloid formation (Faller et al. 2013).

7.6 Therapeutic Strategies for Alzheimer's Disease

Alzheimer's disease and Parkinson's disease are the most common forms of age-related neurodegenerative disorders (Trojanowski et al. 1998). There are still no effective treatments to prevent, halt or reverse Alzheimer's disease, but research advances over the past three decades could change this gloomy picture. Genetic studies demonstrate that the disease has multiple causes. Interdisciplinary approaches combining biochemistry, molecular and cell biology, and transgenic modelling have revealed some of its molecular mechanisms (Huang et al. 2013) Progress in chemistry, radiology and systems biology is beginning to provide useful biomarkers, and the emergence of personalized medicine is poised to transform the pharmaceutical development and clinical trials (Huang et al. 2013). Therapeutic therapy at the molecular level is being targeted at key proteins, which are in AD mechanism such as tau protein, Aβ protein and Apoe4 protein (Huang et al. 2013). Currently drugs target acetylcholinesterase and NMDA receptors are being used to curb the symptoms of AD. Recent studies on plant extracts exhibit promising results, which can be further exploited for finding candidate drugs for Alzheimer's disease. Phytochemicals from families like Araceae, Umbelliferae, etc., are being utilized for decoding pharmacological treatment for AD (Dastmalchi et al. 2007).

7.7 Conclusion and Future Directions

At present, protein folding has turned out to be a spotlight of interest in pharmaceutical investigation: it is feasible that novel approaches to the treatment of diseases such as Alzheimer's are to be found within its intricate pathways. In view of the fact that the number of AD patients is likely to amplify over the next 30 years, huge enhancement in healthcare expenses and care burden due to AD are expected as well. As a consequence, proficient and cost-effective treatment will be main waves for AD research. It is believed that Aβ oligomers could be the intermediates in developmental pathway of amyloid fibril rather than necessary factor in fibril formation. In the most recent years, it is becoming progressively more apparent that not the plaques but Aβ oligomers are the principle pathogenic agent in AD. There are strong evidences in scientific literature that oligomeric intermediates are comparatively more toxic and correlate well with Alzheimer's symptomology than final insoluble Aβ aggregates. Lesser diameter Aβ oligomer multitudes can diffuse into synaptic clefts much easier to induce neuronal and synaptic dysfunction compared with insoluble Aβ plaques.

Since last three decades, scientists have made significant development in understanding malformed brain function in Alzheimer's disease. While novel drugs take years to manufacture from conception to market—and since drugs that appear hopeful in early-stage studies may not work as anticipated in extensive trials—it is essential that Alzheimer's and associated dementias study continue to speed up. Among the majority of vigorously examined categories of possible amyloid inhibitors are natural phenols, an extensive group of plant molecules. Additional information and persuasive clinical trials are essential before these compounds are used for humans. However, these molecules undeniably show strong potential to be effective therapeutic agents against amyloid diseases. These natural molecules may serve dual purpose, viz.; they may be used as tools to study the amyloid aggregation pathway and as molecular scaffolds to widen our knowledge about additional dynamic and biologically obtainable drugs.

Nevertheless, it is expected in near future that numerous drug classes will demonstrate to be proficient and safe intended at undoing the neurodegeneration in AD with the assistance of biomarkers that will foresee expansion of the disease before progression of obvious dementia.

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Part III

Chaperone Surveillance of Proteopathy

Proteopathies: Biological, Molecular and Clinical Perspectives

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Abstract

Importance of proteins and their proper folding in maintaining cellular homeostasis could never be more appreciated than in the current era. Scrutinizing the etiological factors of various debilitating diseases like Parkinson's disease, atherosclerosis, Li-Fraumeni syndrome, cystic fibrosis, diabetes mellitus type 2, etc., that are currently affecting a significant fraction of world human population reveals protein misfolding to be the common thread in all. The cytotoxic symptoms observed in these diseases could be easily explained by genetic mutations directly affecting primary structure and hence conformation of the protein, resulting in either their cellular deficiency due to excessive degradation or accumulation of their cytotoxic oligomers and amyloid fibrils. In this chapter, we have emphasized the expanse of protein misfolding disorders and explained their pathogenesis and pathophysiology. In addition, the medications presently being employed to patients and the recent advances in the development of novel treatment strategies both in primeval and clinical trial stage have been elaborately discussed.

Keywords

Protein misfolding • Proteopathies • Neurodegenerative disorders • Amyloidosis • Aggregation • Molecular chaperone

8.1 Introduction

Current medical and technological advances have increased life expectancy. But, this luxury is eclipsed with the emergence of multiple degenerative disorders like Parkinson's disease, cystic fibrosis, muscular dystrophy, atherosclerosis, diabetes, etc. As prodigious it might sound, these enlarged ensemble of diseases have not stemmed from any pathogenic invasion but rather by imbalanced homeostasis of proteins. It is evidently known that proteins are structural and functional basis of a cell, therefore, their improper folding, overexpression, or excessive loss is found to be detrimental. To avoid such deleterious circumstances, during the course of evolution, cells have evolved an efficient protein quality control system, consisting of chaperones, ubiquitin proteasomal system (UPS), and autophagy. Generation of molecular chaperones is the very first response cells usually show against misfolded protein. It is the responsibility of chaperones to guide and promote correct folding of newly translated polypeptides and prevent their perilous interactions with other cellular proteins and organelles. In spite of that, a large fraction of newly translated protein fails to fold correctly, generating extensible burden of defective polypeptides, which are then directed by the chaperones for degradation either by ubiquitin-proteasome system or autophagy. However, under certain circumstances, this quality control system fails to identify and remove misfolded proteins, leading to formation of soluble or insoluble aggregates, which may accumulate as amyloidogenic plaques (Taylor et al. 2002). These aggregates can then move to different places in the cell and gain toxicity of various natures, leading to different pathological consequences in the cell. Alternatively, overwhelming of this system might also elaborate proteasome mediated enhanced degradation of the protein.

Protein misfolding is now considered the pathological hallmark of a large number of human diseases. Although, this was not the case initially, as earlier abnormal protein conformation was generally linked with just defective enzyme function and related disorders. However, with increasing pace of research in this field, various familial and sporadic diseases are springing out to be the consequence of protein misfolding in almost every tissue and organ system. In the following chapter, we have summarized the protein-related disorders under the following sections, namely – protein misfolding-related neurodegenerative disorders, cardiovascular disorders, metabolic disorders, pulmonary disorders, and

ophthalmic disorders. Also, for the ease of classification under these sections, most of the systemic disorders are kept under the category of other disorders. Through this chapter, we have revised a few disorders of each category and tried to provide the recent developments that have been done in those fields. In the later sections, the understanding gained until now regarding the causes and the mechanisms of toxicity has been elaborated. Finally, the most essential aspect of all these research – the therapeutic advances – has been discussed.

8.2 Proteopathies: The Devil Widespread from Head to Toe

Proteopathies is an umbrella term used to represent the disorders associated with structural abnormality of proteins. Protein misfolding-related disorders are quite prevalent and are extensively investigated in nervous system, but of late they have been found to be associated with diseases localized in almost every organ system. This section provides the detailed overview of representative diseases of various organ systems, describing their clinical manifestations and connection with improper protein folding (See Table 8.1 for list of various proteopathies).

8.2.1 Neurological Disorders

Neurodegenerative diseases (ND) are some of the most debilitating disorders, affecting thinking, skilled movements, feelings, cognition, and memory. The name for these diseases is derived from a Greek word υρο-, néuro, "nerval" and a latin verb dēgenerāre, "to decline" or "to worsen". Despite significant dissimilarities in clinical manifestation, this diverse group of diseases including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), frontotemporal lobar degeneration (FTLD-U), Huntington disease (HD), Prion diseases (PrP), and many more share some common features such as their advent late in life, the widespread neuronal loss and synaptic aberrations, and the existence of cerebral accumulation of misfolded protein aggregates in neuronal inclusions and plaques. These deposits are emblematic signature of most of the neurological disorders and can trigger cascade of events ultimately resulting in synapse loss and neuron death with devastating clinical consequences.

Albeit the differences in the primary sequence of the respective protein in each ND, these proteins have similar morphological, structural, and staining characteristics and are anticipated to have destabilized secondary and/or tertiary structures in vivo and also referred as intrinsically disordered proteins. However in each ND, despite the distinctive localization, distribution, and composition of protein aggregates, cross-β sheets were a common feature in all disease-related amyloids.

8.2.1.1 Alzheimer's Disease

Alzheimer's disease (AD), the most common neurodegenerative disorder, is the late-onset dementing illness, with progressive loss of memory, task performance, speech, and recognition of people and objects. Familial AD has been correlated with mutations of the amyloid precursor protein (APP) gene, presenelin-1 gene, and presenelin-2 gene. These autosomal dominant mutations are all involved in the metabolism of APP, whose cleavage by β-secretase and γ-secretase yield β-amyloid (Aβ) peptides, particularly Aβ1–40 and Aβ1–42 fragments. The Aβ1-40 form is the more common of the two, but Aβ1-42 is more prone to aggregate and is thus associated with disease states. Moreover, presenelin-1 and its homolog presenelin-2 associate with three additional proteins – nicastrin, aph-1, and pen2 – to form a tetrameric complex which functions as γ-secretase (Bi 2010). Sporadic cases of AD seem to be allied with the gene coding for apolipoprotein E (ApoE). Studies have shown the population with ApoE4 allele is at greater risk of developing AD than the general population, while the ApoE2 allele confers relative protection (Farrer et al. 1997). However, the exact nature of ApoE and AD connection is still ambiguous. A few among the other multiple genes whose mutations have been implicated in

Table 8.1 List of proteopathies and the misfolded protein associated with it. The disorders discussed in the chapter are highlighted with bold characters

Disease	Associated protein
Neurodegenerative disorders	
Alzheimer's disease, Cerebral β-amyloid angiopathy	Amyloid-β, Tau protein, ApoE4
Amyotrophic lateral sclerosis	Superoxide dismutase 1
Cerebellar ataxias	Ataxins (polyQ expansion)
Creutzfeldt Jacob disease	Prion protein
Dentatorubro-pallido-Luysian atrophy	Atrophin 1 (polyQ expansion)
Familial amyloidotic neuropathy	Transthyretin
Familial amyloid polyneuropathy III	Apolipoprotein A-1
Familial British dementia	Abri
Familial Danish dementia	ADan, Amyloid-β
Familial encephalopathy with neuroserpin inclusion bodies	Neuroserpin
Frontotemporal dementias	Tau protein
Frontotemporal lobar degeneration	TDP-43
Hereditary cerebral hemorrhage with amyloidosis	Cystatin C
Huntington's disease	Huntingtin (polyQ expansion)
Parkinson's disease and other synucleopathies (multiple)	α-Synuclein
Spinocerebellar ataxia 17	TATA box-binding protein (polyQ expansion)
Spongiform encephalopathies	Prion protein
Ubiquitin positive, tau negative and α-synuclein-negative frontotemporal dementia	TAR DNA-binding protein 43
Metabolic disorders	
Aspartylglucosaminuria	Aspartylglucosaminidase
Congenital sucrase-isomaltase deficiency disorder	sucrase-isomaltase
Diabetes mellitus type 2	Islet amyloid polypeptide
Familial hypercholesterolaemia	LDL receptor protein, Apolipoprotein B
Gaucher's disease	Glucocerebrosidase
Hepatosteatosis	Keratin
I-cell Disease	*N*-acetylglucosaminephosphotransferase
Insulin-related amyloid	Insulin
Lysozyme amyloidosis	Lysozyme
Ornithine transcarbamylase deficiency disorder	Ornithine transcarbamylase
Phenylketonuria	Phenylalanine hydroxylase
Pituitary prolactinoma	Prolactin
Tay-Sachs disease	β-Hexosaminidase

(continued)

Table 8.1 (continued)

Cardiovascular disorders	
Aortic medial amyloidosis	Medin (lactadherin)
Atherosclerosis	Lipoproteins
ATTR amyloidosis	Transthyretin
Cardiac atrial amyloidosis	Atrial natriuretic factor
Desmin-related cardiac myopathy	Desmin, dystrophin
Fibrinogen α-chain amyloidosis	Fibrinogen
Neomaline cardiac myopathy	α-Actin, α- & β-Tropomyosin, Troponin T, Nebulin
Sickle cell anemia	Hemoglobin
Thalasemia	Hemoglobin
Pulmonary disorders	
Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator protein
Emphysema	Alpha-1 antitrypsin
Pulmonary alveolar proteinosis	Surfactant protein C
Ophthalmic disorders	
Cataracts	Crystallins
Corneal lactoferrin amyloidosis	Lactoferrin
Hereditary lattice corneal dystrophy	Keratoepithelin
Retinitis pigmentosa	Rhodopsin
Muscle disorders	
Kennedy disease	Androgen receptor (polyQ expansion)
Muscular dystrophy	Poly (A)-binding protein 2
Sporadic inclusion body myositis	APP, abeta
Short-chain acyl-Coenzyme A dehydrogenase deficiency disorder	Short-chain acyl-CoA dehydrogenase
Neomaline myopathies	α-actinin
Cancer	
Li-Fraumeni syndrome	p53 protein
Medullary thyroid carcinoma	Calcitonin
Retinoblastoma	Retinoblastoma
Epithelial tissue disorders	
Cutaneous lichen amyloidosis	RET
Hypotrichosis simplex of the scalp	Chordiodesmin
Junctional epidermolysis bullosa	Laminin 5
Other disorders	
AL (light chain) amyloidosis (primary systemic amyloidosis)	Monoclonal immunoglobulin light chains
AH (heavy chain) amyloidosis	Immunoglobulin heavy chains
AA (secondary) amyloidosis	Amyloid A protein
Familial Mediterranean fever	Serum amyloid A
Hemodialysis-related amyloidosis	β2-microglobulin
Leukocyte adhesion deficiency disorder	CD18
Zellweger syndrome	Peroxisomal proteins

The disorders discussed in the chapter are highlighted with bold characters
PolyQ polyglutamine

AD pathogenesis are gene for insulin-degrading enzyme, SORL1gene, and ubiquilin-1 (Minati et al. 2009).

AD has been exemplified by the formation of two types of protein aggregates in the brain, senile plaques and cerebral amyloid angiopathy, which are accretions of Aβ peptide (1), and (2) the intracellular neurofibrillary tangles comprised of hyperphosphorylated filaments of the microtubule-associated protein tau. Using NMR studies, Aβ fibrils were revealed to have cross-β structure, a dimer of β-sheets parallel to the fibril axis formed by interaction of β1- and β2-strands of two Aβ peptides. Contradictory evidences from various experiments suggest the pathogenic agents responsible in amyloid-related diseases to be either the transient, prefibrillar Aβ assemblies or oligomers preceding the formation of mature fibrils (Benilova et al. 2012). In both cases, however, the cellular toxicity is believed to manifest through the progression of events such as synaptic failure, inflammation, and tau hyperphosphorylation (Reitz 2012). Furthermore, hyperphosphorylation of tau protein reduces its proficiency to bind with microtubules, bringing about its self-aggregation into paired helical fragments, which then deposits into neurofibrillary tangles. These aggregates of tau protein are believed to further aggravate the toxic insults of Aβ amyloids by (i) triggering synaptic excitotoxicity caused due to enhanced interactions between NMDA receptors and PSD95 proteins (Ittner et al. 2010), and (ii) collapsing the cellular scaffolding to produce widespread brain cell degeneration and dysfunction (Handoko et al. 2013).

8.2.1.2 Parkinson's Disease

Parkinson's disease (PD) is characterized by resting tremor, bradykinesia (slowness of movement), rigidity, and postural instability, and the patients with PD have characteristic hallmarks of loss of dopaminergic neurons and α-synuclein (aSyn) aggregate depositions, also called Lewy bodies, in the cytoplasm of neurons of the substantia nigra (SN) in the brain. aSyn is expressed as multiple isoforms spanning 98-, 112-, 126-, and 140-amino acid residues as a result of alternative pre-mRNA splicing of SNCA gene (also known as PARK1). Variants of SNCA gene, namely A53T, A30P, and E46K, as well as the presence of multiple copies of Wt SNCA gene are related to early-onset familial PD. Genome-wide association studies (GWAS) have linked SNCA with sporadic PD also (Stefanis 2012). Additionally, cohort studies have reported the family with carriers of G51D SNCA mutation to display neuropathological features of both PD and multiple system atrophy, reinforcing the connection of SNCA with PD progression (Kiely et al. 2013). PD is also attributable to mutations in genes other than SNCA, such as parkin, DJ-1, PINK1, and LRRK2.

aSyn is considered to be natively unfolded in its monomeric state as its purified form at neutral pH lacks an ordered structure and function. However, inside cell, they attain alpha-helical conformation after binding with cell membrane. Recent investigations have even reported endogenous aSyn isolated from brain tissue, living human cells, and neuronal and non-neuronal cell lines to exist as a tetramer of about 58 kDa (Bartels et al. 2011). The interesting feature of these tetrameric species was its ability to resist aggregation, suggesting aSyn to disassemble into monomers under diseased conditions prior to its aggregation into pathogenic amyloid fibrils. Structural studies have implicated the central hydrophobic region of aSyn (61–95), so-called NAC (non-Aβ component), to confer it the β-sheet potential upon aggregation. Moreover, investigations have shown aSyn lacking NAC domain has less toxicity and aggregation propensity (El-Agnaf et al. 1998). This aSyn aggregation process prior to the formation of amorphous aggregates, and amyloid fibrils, also involves the formation of various soluble oligomeric intermediates, collectively termed protofibrils, which assume spherical, ring, and string like characteristics when seen under the electron microscope (Rochet et al. 2012). While there are evidences that describe amyloid fibrils to mediate cellular toxicity by disrupting cellular topography and interfering with normal cellular physiology; recent developments have led to the assumption that oligomers/protofibrils are responsible for toxicity in PD. This theory is supported by certain

studies, such as the one done on aSyn variants – A53T and A30P – indicated the neurotoxic effects to be a result of enhanced protofibril formation rather than accelerated fibrillation, and another study done on aSyn variants that promoted oligomer formation also revealed most prominent dopaminergic cell death upon lentiviral injection into rat SN (Rochet et al. 2012). Because of such imperative role of aSyn in PD pathogenesis, the utilization of aSyn levels in colonic mucosal biopsy has been suggested as a biomarker for PD (Shannon et al. 2012). But measurement of only aSyn levels does not seem an efficient and specific biomarker for PD, since AD patients are also known to have significantly increased aSyn levels in the cerebrospinal fluid. However, analysing levels of both aSyn and neurosin, aSyn cleaving enzyme might be a better method to distinguish synucleopathies like PD from AD, since PD patients display lower neurosin and aSyn cerebrospinal fluid levels as compared with non-demented controls and AD patients (Wennstrom et al. 2013).

8.2.1.3 Huntington Disease

Huntington disease (HD) is a fatal disease with symptoms including involuntary movements (chorea), personality, and cognitive changes. HD is a representative of the congregate of polyglutamine repeat (polyQ) disorders, whose other members include Kennedy disease (spinobulbar muscular atrophy or SBMA), dentatorubropallidoluysian atrophy, and six forms of spinocerebellar ataxia (SCA1, 2, 3, 6, 7, and 17). All sharing a common feature of presence of polyQ tract in the respective protein. In case of HD, the CAG trinucleotide repetition lies within exon 1 of the huntingtin gene, encoding the ubiquitously expressed huntingtin (htt) protein. The longer glutamine portion so introduced within htt modifies its native conformation, instigating inclusion formation in the brain. The threshold polyQ length effect observed in HD patients is quite remarkable, in which the polyQ extensions of $\geq$40 leads to disease, whereas $\leq$40 do not. This might be because htt with polyQ extensions $\leq$40 can be degraded by the proteasome, but when the stretches expand >40, proteasome's proficiency to degrade them reduces, leading to their deposition in the nucleus, backing the notion that aggregation of htt with polyQ stretches is responsible for disease manifestation (Scherzinger et al. 1999). The data available up till now has suggested polyglutamine aggregates to have a unique secondary structure with compact β-sheets with interspersed β-turns every nine glutamines, famously known as "polar zippers." The presence of htt inclusions is quite prominent in the striatal neurons and affect mostly the GABA-producing cells, ultimately diminishing the cellular levels of an essential neurotransmitter acetylcholine, hence loss of voluntary muscle control. Additionally, polyQ htt aggregates are believed to impair UPS by engaging with it within the nucleus, disrupt cellular protein homeostasis by recruiting proteins with short polyQ stretches into the nascent htt aggregates, and induce oxidative stress, immune dysfunction, and mitochondrial dysfunction (Ross and Poirier 2004). The polyQ repeats may also perturb htt's physiological function as a transcription factor, altering the configurations of gene transcription. However, evidences from a few experiments had shown that the toxicity of expanded polyglutamine might not be associated with the formation of visible inclusions, while what remained as a common feature of toxicity in all these experimental systems were insoluble molecular aggregates.

8.2.1.4 Amyotrophic Lateral Sclerosis (Lou Gehrig's Disease)

Amyotrophic lateral sclerosis (ALS) predominantly affects the upper motor neurons of the motor cortex, and the lower motor neurons in brainstem and spinal cord, causing muscle weakness, spasticity, atrophy, progressive paralysis, and ultimately death within the few years of the disease commencement. Approximately 90 % of the ALS cases are sporadic in nature and has no known origin, while familial ALS has been found to be associated with mutations of SOD1 gene, TDP gene, FUS gene, and ANG gene (Kiernan et al. 2011). Initially, it was assumed that the mutation of superoxide dismutase (SOD1) reduces its antioxidant activity and enhances the

build-up of reactive oxygen species (ROS) in the cell, causing toxicity. However, studies done on transgenic mice with null SOD1 expression did not showed any motor neurodegeneration, discarding functional loss to be the reason for disease progression (Reaume et al. 1996). Interestingly, Wt SOD1 overexpression in mice displayed ALS-like pathology and deposition of SOD1 aggregates in brain, suggesting another pathway for ALS development (Graffmo et al. 2012). In fact, the presence of hyaline inclusions immunoreactive for SOD1 in the neurons and astrocytes is the common manifestations of familial and sporadic ALS (Ivanova et al. 2014). It is supposed that either aggregates or oligomeric precursors of mutant SOD1 disturb cellular proteostasis by disrupting protein quality control system and induces stress by interfering with cellular cytoskeletal transport network (Chattopadhyay and Valentine 2009). Current advances have shown that apart from SOD1, the mutations of profilin 1 (protein essential for actin polymerization) may also have an involvement in ALS development by enhancing axonal retraction and denervation in the adult neuromuscular system (Robberecht and Philips 2003). Additionally, in the spinal cord samples of ALS patients, the upregulation of inflammatory cytokines, such as TNFα, COX-2, and interleukins, has been observed (Wijesekera and Leigh 2009). However, the association between inflammatory network and ALS is yet not understood and needs to be explored.

8.2.1.5 Prion Diseases

Prion protein (PrP) diseases, also termed transmissible spongiform encephalopathies (TSEs), are a heterogeneous group of disorders that are characterized by ataxia, myoclonus, dementia, insomnia, and psychiatric disturbances. There are both sporadic PrP diseases, such as sporadic Creutzfeldt-Jakob disease (CJD), as well as familial forms including familial CJD, Gerstmann-Straussler-Scheinker disease, and fatal familial insomnia. Pathologically these disorders are distinguished from other ND by their resemblance with viruses to propagate the disease from infected cell to healthy cells. However, in this case, the entity showing infectious nature is prion proteins, and its variants and their amyloid plaques cause the disease (Aguzzi and Calella 2009). The mutations of PRNP, the gene encoding prions, have been found to be associates with both familial and sporadic prion diseases. The known-disease causing variants of PRNP are Met129Val and Glu219Lys. Genome-wide association studies have also revealed the presence of an additional candidate loci upstream of the retinoic acid receptor beta encoding gene to be associated with CJD (Mead et al. 2009).

In PrP diseases, prions undergo conformational changes from its normal conformation, PrP^{C} to its insoluble protease resistant pathological form, PrP^{Sc}. Findings accumulated in last three decades via various studies such as understanding prion disease manifestation in Wt animals injected with highly purified PrP^{Sc} and finding that PrP knockout mice are resistant to prion infection has given rise to the prion hypothesis (Soto and Estrada 2008). This hypothesis explains the mechanism of conversion of PrP^{C} into PrP^{Sc}. According to the hypothesis, only PrP^{Sc} not PrP^{C} has the ability to replicate in the brain without requiring nucleic acids, and it catalyzes the conversion of Wt PrP^{C} into the misfolded form. It is believed that oligomeric PrP^{Sc} acts as a nuclei to bind PrP^{C}, perhaps in combination with certain ancillary proteins, and incorporate it into the growing oligomer. However, at some point, the long PrP^{Sc} polymers break into smaller pieces, which then further act as seeds to drive PrP^{C} conversion, producing a self-propagating amyloid. Interestingly, a recent study done with spleen tissue from a PRNP heterozygous genotype individual had demonstrated to propagate the variant CJD agent, and it was realized that the prions can be present in the other tissues without CNS involvement (Bishop et al. 2013). This development furthers that despite multitudes of investigations, our knowledge regarding this disease is still limited and it is possible that prion disorders affect multiple tissues not just nervous system, increasing the challenge to find its cure.

8.2.1.6 Are All Neurodegenerative Proteopathies Infectious?

PrP diseases have exemplified that how protein's conformational change can make a protein to behave like a pathogen and cause an infectious disease. This infectious nature of prions is best explained by seeding-nucleation model as the disease progression lie on the competence of preformed stable misfolded oligomeric proteins to act as a seed to catalyze the misfolding and aggregation process. It is quite possible that the proteins involved in other neurodegenerative disorders also have yet undiscovered ability to be transmissible, since their aggregation and amyloidosis also follow the same model as that of prions. With recent advances, various reports have arose that defends this hypothesis. Aggregates of Aβ peptide have been reported to spread to unaffected cells when injected into the brain of an AD mouse and display Aβ accumulation in a pattern dependent upon both the host and the agent (Rosen et al. 2011). The capability of passing between living cells was also illustrated for aggregates of truncated tau, consisting of the microtubule-binding region and a fluorescent protein tag that can leave and enter cells in culture, and promoted the aggregates and fibrillization of normal tau within them (Jellinger 2011). Another study has reported that tau protein aggregates to spread around neighboring areas of the brain by "jumping" within neurons (Brundin et al. 2010). The various studies done on neuronal cell lines, animal models, and humans, where PD host with grafted dopaminergic neurons shows lewy body pathology, has reinforced the concept that neuronal protein aggregates can display prion-like pathogenic behavior (Angot et al. 2012). This is reflected in propagation pattern of aSyn-rich lesions, in accordance with Braak hypothesis of staging of PD, first through the lower brainstem, the anterior olfactory nucleus, and olfactory bulb, and then subsequently in mesencephalic and neocortical regions. Lately, a groundbreaking work describing the first ever research model that shows both cell-to-cell spread of aSyn aggregates and progressive loss of dopaminergic neurons has reinforced the former hypothesis. In this investigation, single injection of preformed fibrillar aSyn into the striatum of Wt mice was sufficient to induce intraneuronal aSyn accumulation and Lewy body pathology (Luk et al. 2012). The propagation of proteinaceous lesions has also been demonstrated in aggregates of SOD1, TDP-43, and polyQ proteins (Jellinger 2011). The presence of prion-like domain in these proteins has been alleged to influence the disease progression. It is necessary to understand that lack of experimental confirmation for infectious nature of protein aggregates in other ND does not mean that it is a disease-specific phenomenon, and the seeding activity displayed by protein aggregates in all neuronal proteopathies clearly indicates cell-to-cell transmission to be general dominion of amyloids.

8.2.2 Metabolic Disorders

8.2.2.1 Diabetes Mellitus Type 2

Type 2 diabetes (T2DM) is a loosely defined clinical syndrome that is characterized by insulin resistance, defective insulin secretion, and loss of β-cell mass (number of β-cells) with increased β-cell apoptosis and islet amyloid deposition. The amyloid lesions within the islets are depositions of misfolded islet amyloid polypeptide (IAPP) oligomers with cross-β sheet structure (Hayden et al. 2005; Patel et al. 2014). The initial site of localization of these lesions in the islets is not known. However, the most probable site seems to be insulin secretory granule (ISG), the storage reservoir of pro-insulin and pro-IAPP. It is here prohormone convertases 1, 2, and 3 process pro-IAPP into their cleaved forms, which are then coreleased into the circulation (Marzban et al. 2004). Any abnormality in this processing has been observed to ensue IAPP-derived islet amyloid deposition, demonstrating pro-IAPP also to be amyloidogenic (Hayden et al. 2005). Imbalance among other components of ISG, such as Ca^{2+}, Zn^{2+}, and C-peptide, that maintain mature insulin and IAPP is also believed to promote aggregate formation (Westermark et al. 1996). Additionally, excessive ROS burden and ER stress in conjugation

with excessive insulin production increase protein misfolding, thereby overwhelming cell's PQC and fostering IAPP aggregation (Back and Kaufman 2012). Other than IAPP and insulin, this islet amyloid also contains serum amyloid P component (SAP), ApoE, and the heparan sulfate proteoglycan, perlecan. Perlecan stabilizes islet amyloid by allowing IAPP to bind to the basement membranes surrounding islet capillaries, resulting in ISG's decreased secretory response due to thickened basement membrane (Kahn et al. 1999). This event is supposed to activate the structural alterations within islet, thus creating a localized milieu with predisposition for skewed insulin to IAPP ratio.

The physiological function of IAPP is yet not fully apprehended; however, studies with rodent models have alleged its involvement in the regulation of various metabolic constraints including satiety, gastric emptying, adipose accumulation, and blood glucose levels via glucose-simulated insulin secretion inhibition (Abedini and Schmidt 2013). This loss of physiological function could be the additive factor in the toxicity seen in T2DM. However, oligomerization of IAPP seems to be the major contributor for apoptosis of β-cells via instigation of localized inflammation in islet, oxidative stress enhancement, and autophagy dysregulation.

8.2.2.2 Phenylketonuria

Phenylketonuria (PKU), the first treatable inherited disorder, is an inborn error of metabolism of L-phenylalanine (L-Phe) caused by mutations of the gene encoding phenylalanine-4-hydroxylase (PAH). At present, approximately 500 mutations of PAH gene are known, most of whom are found to be associated with PKU. These mutations either leads to degradation or aggregation of misfolded PAH enzyme, ultimately causing its deficiency in the liver. Since PAH with cofactor tetrahydrobiopterin is involved in the catalysis of L-Phe to L-tyrosine, its deficiency reduces tyrosine levels and elevates blood levels of L-Phe and its metabolites, impairing brain development and function (Donlon et al. 2004). Depending upon the L-Phe levels and loss of PAH activity, PKU is differentiated into various categories – classic PKU with slight or no activity (Phe > 1,200 μmol/l), mild PKU, and non-PKU hyperphenylalaninemia with some activity (Phe > 360–1,200 μmol/l and 120–600 μmol/l, respectively) (Williams et al. 2008).

Although unlike other protein misfolding disorders PKU is treatable, the lifelong dietary control often leads to under nutrition and psychosocial problems (Harris 2014). Therefore, in order to create therapeutic interventions disrupting PAH oligomerization, it is necessary to understand its conformational dynamics during catalysis and aggregation. The investigations done so far have not much advanced our knowledge, what we know is that PAH is a homotetramer, which on binding with L-Phe and tetrahydrobiopterin alters its conformation and gets activated. These structural changes involves fine interplay among PAH monomers and various domains via networks of side-chain interactions. This conformational flexibility is believed to make PAH vulnerable to misfolding. This is buttressed by the studies displaying unstable aggregation and enhanced degradation of variant PAH enzymes (Gersting et al. 2008).

8.2.3 Cardiovascular Disorders

8.2.3.1 Atherosclerosis

Atherosclerosis is a progressive vascular disease characterized by chronic inflammation, augmented oxidative stress, and arterial vessel thickening due to atherogenesis and amyloid plaque formation. Smoking, diabetes mellitus, imbalanced lipid metabolism, high blood pressure, and enhanced cholesterol levels are some of its known risk factors (Ursini et al. 2002). The prolonged inflammation observed in atherosclerotic arteries is a general immune system response to injury, but in this case it further aggravates the condition by expanding arterial plaque and narrowing the vessels (Herczenik and Gebbink 2008). Arterial vessel narrowing, if untreated, leads to various cardiac complications for instance, stroke, peripheral arterial disease, stenosis, and myocardial infarction (Wilck and Ludwig 2014). Additionally, rupturing and release of arterial

plaque components in blood could cause embolism and prove fatal (Herczenik and Gebbink 2008). Atherosclerotic arterial lesions are generally comprised of cholesterol, calcium, lipoproteins, macrophages, and aggregates of various misfolded proteins such as Aβ, α_1-antitrypsin, and members of apolipoprotein (Apo) family (Rocken et al. 2006). Apo proteins are essential for lipid metabolism, since their binding is necessary for the activation of various physiological lipid and cholesterol carriers such as high density lipoprotein (HDL), low density lipoprotein (LDL), etc. Oxidation of LDL has been reported to alter its native conformation and convert α-helix to β-sheets, thereby priming LDL fibrillation and amyloidosis (Maor et al. 1997). However, recent investigations done with 17-β-estradiol showed its binding with modified apoB-100 to inhibit the misfolding and hence aggregation of LDL and apoB-100 complex, suggesting apoB-100 misfolding to be the root cause behind amyloid formation and atherogenesis (Brunelli et al. 2014). Unlike most of the other misfolded proteins, structure destabilization or proteolysis does not seem to be the reason for the deposition of mutated apo protein fibrils in atherosclerotic lesions. This hypothesis is conferred from the structural analysis of apoA-1 variants, which displayed very minute changes in protein stability, α-helical content, protein-lipid interactions, and proteolytic pattern (Das et al. 2014).

8.2.3.2 ATTR Amyloidosis

ATTR amyloidosis is the most common form of familial cardiac amyloidosis, caused by mutation in the gene for the plasma protein transthyretin (TTR), and is characterized by severe heart failure and arrhythmias. Out of the 100 different amyloidogenic missense point mutations designated for TTR gene, V122I is the most frequently observed variant. This mutation was found in 3.9 % of all Afro-American population and 23 % of African Americans with cardiac amyloidosis and may be responsible for the higher prevalence of heart failure in elderly blacks (Buxbaum et al. 2006). The onset and severity of disease manifestation vary with specific mutation. For instance, TTR with Val30Met mutation is rarely corresponded with amyloid development in cardiomyocytes, while another TTR variant with Thr60Ala mutation develops cardiac amyloidosis early in individuals (Banypersad et al. 2012). It is assumed that mutant TTR act as nuclei and prompts aggregation of Wt TTR. This was braced by the study done in Japan, which reports Wt TTR to be present in the hearts of patients with familial amyloid polyneuropathy (Kholova and Niessen 2005).

8.2.3.3 Aortic Medial Amyloidosis

Aortic medial amyloidosis is a very common protein deposition disease of aortic media in elderly people. It is characterized by the presence of amyloid fibrils composed of medin protein. Medin is a 50-residue fragment derived from internal splicing of lactadherin, a milk fat globule protein (Stubbs et al. 1990). This disease has no well-established clinical significance but is considered to be involved in the age-related loss of elasticity of the vessels. Not much research is yet done on this aspect of this disorder because of which the cause of misfolding and the mechanism of toxicity are not clear.

8.2.4 Pulmonary Disorders

8.2.4.1 Cystic Fibrosis

Cystic fibrosis is one of the most widespread life-shortening inherited disorders among the caucasian population and is characterized by airway infection, inflammation, remodeling, and obstruction, resulting in gradual destruction of the lung tissue and ending in an early death. It follows autosomal recessive inheritance as the disease develops only when both copies of cystic fibrosis transmembrane regulator (CFTR) gene are mutated. CFTR is a cAMP-activated ATP-gated anion channel that transports chloride and thiocyanate ions across epithelial cell membrane to the covering mucus. To maintain electrical balance, sodium ions also depart from the cell, which leads to increased osmolarity of the outer mucosal cells, ensuing water to move out of cell by osmosis. All disease-causing mutations in the CFTR gene prevent the protein from attaining

proper conformation and, hence, are retained and degraded. The loss of CFTR function impedes the CFTR channel from functioning properly; inhibits the movement of ions and water out of the cells; causes the development of thick, sticky mucus which then cause obstruction of the passageway; and traps bacteria that give rise to chronic infections (Childers et al. 2007).

CFTR protein quality control is mediated at either of the two stages: (a) Molecular chaperone, heat shock protein (Hsp) 70 stops the misfolded protein processing in the cytosol and present it to the proteasome for degradation; (b) Calnexin, a lectin chaperone of ER bind to glycosylated CFTR and retro-translocates the misfolded CFTR to the cytosol for degradation (Gregersen 2006). In addition, the study done in ΔF508 CFTR yeast model (the most common CFTR variant) had reported human small Hsp, αA-crystallin to preferentially interact with ΔF508 CFTR, maintain its solubility and direct them for ER associated degradation (Ahner et al. 2007). All these studies suggest differential effect of various chaperons on variant CFTR. This is backed by the investigation done on mutant human cystathionine beta-synthase, where the ratio of Hsp70 and Hsp26 was demonstrated to determine the fate of misfolded proteins to be either refolded or degraded (Singh and Kruger 2009). This model for cystic fibrosis pathology is supported by the study in which ΔF508 was observed to have increased sensitivity to proteolytic digestion, as compared with the Wt CFTR (Bellotti and Chiti 2008). Interestingly, CFTR overexpression or proteasome inhibition has been demonstrated to augment the accumulation of Wt and ΔF508 CFTR protein along with ubiquitin and certain chaperones to develop aggresomes that are surrounded by collapsed intermediate filament proteins, expounding CFTR as an aggregation prone protein under stress conditions (Kopito 2000). Genome-wide association studies have indicated another protein, interferon-related development regulator-1, to be associated with cystic fibrosis. A recent study done to understand its role in disease pathology found its upregulated levels in neutrophils to be linked with enhanced ROS generation (Hector et al. 2013). Further studies are required to clearly understand its role in disease pathogenesis.

8.2.4.2 Emphysema

Emphysema is part of a group of conditions termed chronic obstructive pulmonary disease and is characterized by destruction of lung tissue around the alveoli. Smoking is considered the major causative factor, followed by α1-antitrypsin (AAT) deficiency as the rare cause for emphysema. AAT is a monomeric secretory protein synthesized most abundantly by hepatocytes. After secreting from cells, Wt AAT binds to and inhibits trypsin and also the blood protease, elastase. In the lungs of individual with AAT deficiency, elastase and trypsin degrade the lung tissue that participates in the absorption of oxygen, eventually leading to breathing problems. The reason for AAT deficiency in lungs is the mutation in the gene encoding AAT, causing the production of misfolded protein which is then retained intracellularly and degraded in pre-golgi, non-lysosomal compartment (Stoller and Aboussouan 2005). The study done with transport-impaired PiZ variant (most common variant linked with AAT deficiency) in transfected mouse hematoma cell line demonstrated that these variants form stable soluble aggregates, possibly in the form of homotrimers, which then undergoes a discrete size reduction and degradation within the ER (Le et al. 1992). Severe cases of AAT misfolding may also lead to cirrhosis of the liver, where the AAT aggregates develop a fibrosis that produces scarring and dysfunction.

8.2.4.3 Pulmonary Alveolar Proteinosis

Pulmonary alveolar proteinosis is a well-known conformation disorder characterized by the presence of amyloid fibrils comprising lung surfactant protein C (SP-C) within the alveoli, interfering with gas exchange (Gustafsson et al. 1999). A latest cohort study done in Japan has reported elevation in its incidence and prevalence rate and discarded its linkage with smoking, one of the common causes of lung diseases (Inoue et al. 2008). Variants of gene encoding SP-C have been correlated with chronic lung disease in children and adults. These mutations are

believed to induce pro SP-C protein misfolding, which then cause toxicity in epithelial cells either via its deposition in lungs or pro SP-C trapping in the ER and its rapid degradation via UPS (Bridges et al. 2003). Additionally, fatty acid removal from SP-C is known to induce conformational change, such that it will aggregate to form β-sheet-rich amyloid fibrils (Brasch et al. 2004).

8.2.5 Muscle Disorders

8.2.5.1 Congenital Myopathies

Congenital myopathies include various inborn skeletal muscle disorders frequented with depositions of protein fibrils also called rod bodies. Approximately 45 varied types of myopathies are known and classified on the basis of histopathologic characteristics (Bodensteiner 2014). Due to rare occurrence, not all of them are investigated in great depth. However, core-rod myopathy, reducing body myopathy, cap disease, cylindrical spirals myopathy, and nemaline myopathy have been prominently studied. They don't display any significant morphological differences because skeletal muscle could conceive only limited amount of pathological modifications. So, the major differences observed in their pathogenesis are believed to be because of the associated genetic mutations and related modified proteins. Recent studies have reported mutations in various genes including TPM3, FHL1, TPM2, ACTA1, RYR1, KLHL40, and NEB to be involved with protein inclusion formation (Malfatti et al. 2014; Wilding et al. 2014). These rod bodies generally include fibrils of various proteins, such as troponin T, nebulin, α- and β-tropomyosin, and α-actin.

Nemaline myopathy is a representative member of this diverse group of disorder. Investigations done with cardiac myocytes have confirmed accretions of α-actin fibrils in the sarcomere nucleus as its pathological hallmark (Clarkson et al. 2004). It is assumed that missense mutations in α-actin gene cause structural alterations in the protein in such a way that their tendency to self-associate enhances (Vang et al. 2005). Overexpression of variant α-actin proteins in cultured fibroblasts and myoblast cell lines was also observed to trigger nuclear fibrillation (Costa et al. 2004). The cytotoxicity observed is believed to be a cumulative consequence of loss of sarcomeric actin fibers and generation of toxic protein aggregates (Gregersen 2006).

8.2.5.2 Muscular Dystrophy

Muscular dystrophy (MD) is an ensemble of genetic disorders exhibiting progressive skeletal muscle weakness, hampered locomotion, muscular tissue degeneration, and accretions of defective proteins (Emery 2002). There exist several forms of MD, such as Duchenne, limb-girdle, myotonic, distal, Becker, Emery-Dreifuss, facioscapulohumeral, and oculopharyngeal muscle dystrophies, each affecting different muscle assemblies with manifestations in multiple organ systems. Congenital MD is generally associated with mutations of genes encoding for proteins involved either in connecting muscle tissue with extracellular assembly or in forming dystrophin-associated protein complex (Emery 2002). Duchenne and Becker forms of congenital MD are characterized with the presence of misfolded or truncated dystrophin protein, causing deficiency of active dystrophin and dystrophin glycoprotein complex (Koenig et al. 1989). Dystrophin act as a mechanical stabilizer, and as a part of dystrophin glycoprotein complex, it helps linking cytoskeleton of each muscle cell to the extracellular matrix via sarcolemma (Hoffman et al. 1987). Oculopharyngeal MD is characterized by the presence of polyalanine tract in the nuclear poly (A)-binding protein 1 (PABPN1) and musculoskeletal symptoms like limb weakness, ptosis, and dysphagia. Studies done with cellular and transgenic mouse models of oculopharyngeal MD have revealed the presence of aggregates of protein PABPN1 with polyalanine extensions in the myonuclei of skeletal muscles (Brais et al. 2014). Interestingly, both Wt and mutant PABPN1 are susceptible to aggregation, but only oligomers of mutant PABPN1 are believed to cause cytotoxicity (Raz et al. 2011). Furthermore, reduced availability of functional PABPN1 aggravates the condition by impairing

gene expression, since it is responsible for polyadenylating pre-mRNAs (de Klerk et al. 2012).

8.2.6 Ophthalmic Disorders

8.2.6.1 Retinitis Pigmentosa

In patients with retinitis pigmentosa, the functionality of the retina is affected which can lead to severe visual impairment and blindness. Studies have revealed its most common etiology to be mutations in rhodopsin gene, which leads to the generation of unstable opsin protein that can't bind with its cofactor 11-cis retinal to form functional rhodopsin protein (Anukanth and Khorana 1994; Rosenfeld et al. 1992). Rhodopsin is the pigment found in the rod cells that enables our vision during low-light conditions (Stuart and Birge 1996). The variant opsin protein has an inherent instable structure, which triggers its self-association and binds with Wt opsin proteins to form cytotoxic aggregates (Chen et al. 2014b). These aggregates are resistant from UPS mediated degradation and thus block cellular proteasomes, which incites the production of intracellular inclusions or aggresomes comprising Wt and mutated rhodopsin, certain opsin binding proteins, and other misfolded and unfolded proteins that were destined to be degraded (Saliba et al. 2002). Retinal cells require a rapid protein turnover for its proper functioning, and PQC failure tremendously enhances the misfolded protein load, aiming the cell towards programmed cell death.

8.2.6.2 Cataract

Cataract or opacification of eye lens is one of the most common causes of blindness and has been associated with mutations in the genes encoding for crystallin proteins (Kosinski-Collins and King 2003; Xi et al. 2014). α and βγ Crystallins are the major structural proteins of eye lens responsible for maintaining transparency and enhancing its refractive index (Mahendiran et al. 2014). Additionally, α crystallin functions as molecular chaperone and assist unfolded or misfolded proteins toward degradation, thereby helping lens in tolerating detrimental effects of protein aggregates at old age (Cheng et al. 2010). Mutations in these proteins lead to unobstructed generation of cytoplasmic inclusions, which lead to enhanced light scattering and hence visual impairment (Moreau and King 2012). However, genetic studies done with families displaying juvenile onset cataract have suggested accumulation of mutant γ-crystallin in nucleus to ensue toxicity through disruption of transcriptional processes and structural disorganization of lens fiber cell nucleus (Stefani 2004).

8.2.7 Cancer

Millions of people die every year due to cancer, making it the most fatal disorder worldwide (Stewart and Wild 2014). The current treatment strategies, including radiation, chemotherapy, and ultimately surgical removal of affected organs, are not helpful for patients in last stages of cancer. The immortality and metastasizing features of cancerous cells give them an edge over other pathogens, complicating our comprehension of their pathogenesis and pathophysiology. During malignancy, proteins are usually uncontrollably overexpressed or structurally affected because of genetic mutations, resulting in changes in activity and protein-protein interactions in cancer cells (Xu et al. 2011). Despite this, only recently, role of protein aggregation has been implied in cancer and it has been found that many cancers are associated with the abnormal accumulations of aggregates of Wt and mutant p53. p53 is a tumor suppressor nuclear phosphoprotein that triggers cell cycle arrest and apoptosis in response to cell stress. It is comprised of structured, partially folded and natively unfolded segments, which give high conformational flexibility (Stefani 2004). Over 50 % of reported cases of human tumors are found to be associated with p53 mutations. According to IARC TP53 Mutation Database, over 95 % of the malignant mutations occur in the DNA-binding domain of p53, where they cluster in so-called hot spots of mutation (Olivier et al. 2002).

Nuclear and cytoplasmic aggregates of inactive conformational variant of p53 have been described to be associated with tumors such as retinoblastoma, neuroblastoma, colon cancer,

and breast cancer (Stefani 2004). Various independent studies done in the last decade have shown that several regions of p53 have the tendency to form β-sheet-rich fibrillar aggregates under physiologic and stress conditions. Moreover, mutant p53 had been demonstrated to colocalize with amyloid-like protein aggregates in cancer biopsies (Levy et al. 2011). Like other misfolded proteins, p53 folding variant also has the ability to drive conformational change in Wt p53 and its paralogs p63 and p73. This has been supported by a recent study, where amyloid fibrils of R248Q, a p53 mutant, were reported to seed the aggregation of Wt p53 in a prion-like fashion (Xu et al. 2011). All these evidences imply that p53 lead to tumor genesis either via loss of the antitumor function or by a gain of toxic function.

8.2.8 Epithelial Tissue Disorders

8.2.8.1 Hypotrichosis Simplex of the Scalp

In the last decade, hypotrichosis simplex of the scalp (HSS), an autosomal-dominant form of isolated alopecia, has been recognized as a proteinopathy, which can be characterized by the amyloid-like aggregates of corneodesmosin. Corneodesmosin is a glycoprotein that is expressed in the epidermis and in the inner root sheath of hair follicles (Jonca et al. 2002). It is believed to behave as a keratinocyte adhesion molecule and is encoded by CDSN gene. *In vitro* and *in vivo* studies reported mutations in CDSN to induce toxicity in hair follicles and trigger HSS by causing abnormal proteolysis of Wt corneodesmosin, which results in truncated fragments that aggregate to form oligomers in the superficial dermis and the periphery of hair follicles (Levy-Nissenbaum et al. 2003).

8.2.9 Other Disorders

8.2.9.1 Immunoglobulin Light Chain Amyloidosis

Immunoglobulin light chain amyloidosis is a clonal but non-proliferative plasma cell disorder, illustrated by the pathologic production of fibrillar proteins containing monoclonal light chains which deposit in various tissues (Rosenzweig and Landau 2011). Clonal population of bone marrow plasma cells that produces a monoclonal light chain of κ or λ type either as an intact molecule or a fragment is the characteristic feature of this disorder (Gertz 2013). Like other proteopathies, in this case also, the light chain Ig protein misfold forms an insoluble β-pleated sheet rich protein deposits which then cause organ dysfunction. In the absence of clonal plasma cells in the bone marrow, light chain amyloid may localized to a single site, most often the skin, larynx, or urinary tract (Rosenzweig and Landau 2011). The clinical features indicative of light chain amyloidosis are nonischemic cardiomyopathy with "hypertrophy" on echocardiography, nondiabetic nephrotic syndrome, chronic inflammatory demyelinating polyneuropathy, weight loss, hepatomegaly, increased alkaline phosphatase value with no imaging abnormalities of the liver, edema, or paresthesias (Gertz 2012). The light chain origin of an amyloid deposit can be confirmed with immunohistochemistry or immunogold assay (Gertz 2011). Current treatment approaches for light chain amyloidosis focus on eradication of the monoclonal plasma cell population and suppression of the pathologic light chains which can result in organ improvement and extend patient survival (Rosenzweig and Landau 2011).

8.3 Common Origin of Proteopathies

The clinical symptoms realized in almost all proteopathies are either because of cellular protein deficiency, owing to its enhanced degradation, or because of misfolded protein fibrils and oligomer mediated cytotoxicity. However, in certain cases, impaired protein quality control system is the underlying cause for protein enhanced degradation or accumulation of unfolded proteins, thereby triggering proteopathies. These common origins have been discussed in detail in the following section.

8.3.1 Impairment of Protein Quality Control System

Protein quality control (PQC) system is formed by refined alliance between molecular chaperones localized in cytosol (prefoldin, crystallins, heat-shock proteins, Hsc70) and ER (Grp94, Bip, calnexin), and PQC system including UPS and autophagy. Chaperone-targeted proteolysis is aimed at maintaining protein homeostasis by clearing short-lived or mislocated Wt proteins, overexpressed proteins, proteins that are unable to attain their native conformation due to mutations or posttranslational modifications or interaction with other proteins, damaged proteins that could harm cell, and protein aggregates. Such proteins are initially polyubiquitinated via multienzymatic reactions and are then sent to proteasome for degradation. A recent study done with cardiac proteasomes has reported its different subtypes to have varied susceptibility toward proteasome inhibitors, suggesting that proteasomes are differentially composed and modified to degrade different families of substrates (Kloss et al. 2010). Autophagy provides clearance route for many cytosolic misfolded proteins via micro autophagy, macroautophagy, and chaperone-mediated autophagy. Chaperone-mediated autophagy involves binding of misfolded or unfolded proteins with the receptor protein present on the lysosomal membrane, thereby triggering autophagosome formation, which then moves into lysosomal lumen for degradation. Yeast and cell line model studies have suggested preferential deposition of terminally aggregated proteins like mutant htt and PrP^{Sc} in perivacuolar inclusions (Kaganovich et al. 2008).

Impairment or overwhelming of PQC due to aging, oxidative stress, inflammation, blockage by misfolded proteins, etc. can lead to accumulation of abnormal proteins in cell, causing eccentric inclusion formation furthering cellular damage. For instance, atypical PQC is a common occurrence in cardiac diseases including hypertrophy, heart failure, cardiomyopathy, and atherosclerosis. This is supported by the consensus of the chaperone studies which gave the impression that chaperone's loss of activity compromises heart's ability to handle stress, whereas chaperone's presence prevents from cell injury (Su and Wang 2010). Another example that explains the role of PQC impairment in development of protein misfolding diseases is of familial ALS, which is also caused due to failure of UPS to degrade polyubiquitinated, misfolded SOD1. This conception is backed by the discovery of familial ALS-linked mutations in genes encoding for ubiquilin 2, phosphatidylinositol 3,5-bisphosphate-5-phosphatase, charged multivesicular body protein 2b, valosin-containing protein, and optineurin, since all these proteins are directly involved in protein homeostasis (Robberecht and Philips 2003).

However, certain terminally aggregated proteins are resistant to proteasomal degradation; as in the case of HD, proteasome's active site is incapable of cleaving polyQ extensions of mutant htt, thereby preventing its clearance from the cell (Palombella et al. 1989). Furthermore, current developments done towards understanding the mechanism underlying retinal dystrophies such as McKusick-Kaufman syndrome, Leber congenital amaurosis, retinitis pigmentosa, and Bardet-Biedl syndrome have revealed their appearance to be associated with mutations in the gene encoding opsin chaperones RP2, MKKS, and AIPL1 (Chapple et al. 2001). All this data clearly suggests a prominent role of PQC system in preventing the development of various proteopathies. Unambiguous understanding of the PQC modulation pathways would reveal several therapeutic targets.

8.3.2 Enhanced Degradation of Proteins

The highly efficient protein quality system with the intention to avoid development of antigenic or misfolded proteins degrades significant number of proteins prior to their maturation. This protects the cell from harmful protein inclusions and proves advantageous against pathogenic infections, where fragments of non self-proteins degraded via UPS are presented as antigen to prompt immune system. But, this stringent

scrutiny of PQC sometimes could prove detrimental. For instance, the deletion mutants of gene encoding α-subunit of the lysosomal enzyme β-hexoaminidase associated with Tay Sach's disease expresses truncated protein, which being rejected by PQC becomes deficient in the cell, and triggers associated clinical symptoms (Lau and Neufeld 1989). The functional loss of mutated proteins because of its retention and enhanced degradation in the ER by cell's PQC is a common etiology for various other disorders including emphysema, cystic fibrosis, congenital sucrase-isomaltase deficiency, etc.

Sometimes, pathogens and misfolded proteins prevent themselves by utilizing host's PQC against them, for instance E6 genes of both high-risk and low-risk human papilloma viruses (HPV) have been reported to immortalize human mammary epithelial cells by inducing degradation of p53 protein *in vivo* (Band et al. 1993). Similarly, HPV-16 E7 protein was found to enhance the degradation of retinoblastoma protein as an additional mechanism of oncogenic transformation (Boyer et al. 1996). Furthermore, a recent investigation done to understand the contribution of ApoE in sporadic AD revealed ApoE to promote β-amyloid degradation by modulating microglial cholesterol levels (Lee et al. 2012).

8.3.3 Aggregate/Amyloid Formation

Proteins when not properly folded and prevented from cell's refolding or degradation mechanisms start intramolecular interactions with other unfolded proteins via their exposed hydrophobic regions, spontaneously leading to their aggregation. It is generally known that mutations, genetic alterations, or hostile solvent (rarely) are the basic cause of protein misfolding. Most of the disease-causing genetic alterations do not allow protein to fold into its lowest free energy native state, thereby accumulating non-functional protein intermediates which are either degraded by cell's PQC or aggregate to form fibrils. Initially, these intrinsically trapped species aggregate to form soluble oligomers, which then further self-associate to form insoluble amyloid fibrils. Amyloids are ordered aggregates having no universal tertiary or quaternary structure, but are enriched with cross beta-sheet structure with the spasmodic presence of stabilizing rows of hydrophobic interactions that run along the fibril axis (except in case of htt). Under electron microscope, they appear as unbranched fibrils with a consistent diameter of 7.5–12 nm and give apple-green birefringence with histological dyes such as congo red. The process of amyloid fibril formation follows nucleated growth mechanism, which includes a lag phase followed by a rapid exponential growth phase of fibril formation. Lag phase or the time required by peptides to form "nuclei" is the rate-limiting step, while growth phase is the time required by monomers and oligomers to irreversibly associate with the core. However, certain mutations and addition of preformed fibrillar species are known to decrease or eliminate the lag phase, indicating that in these cases amyloid formation is no longer dependent on the formation of a nucleus. Since this process follows second order kinetics, the initial concentration of misfolded proteins is an essential factor to determine its fate.

The eccentric attribute of aggregation prone proteins is their structural flexibility, which enhances their tendency to self-associate in the presence of external stimulants such as mutation, local environment, protein cross-linking with transglutaminase, redox changes, and posttranslational modifications. Presence of phosphorylated, nitrated, and oxidized forms of aSyn in the Lewy bodies and hyper-phosphorylated tau protein in neurofibrillary tangles has suggested an essential role of posttranslational modifications in modulating fibrillation. This is supported by the studies done on fly and mouse models of SCA, where blockage of Ataxin-1 phosphorylation was observed to reduce the extent of inclusion formation and degeneration of purkinje neurons (Ross and Poirier 2004). Prion proteins have been also reported to adopt many of the physical characteristics of PrP^{Sc} after posttranslational modifications (Dear et al. 2007). Furthermore, sumoylation of htt protein is believed to decrease its ability to aggregate and

increase neurodegeneration by competing with ubiquitin for binding at identical target lysines, preventing its degradation by proteasome (Steffan et al. 2004).

Role of hydrophobicity of amino acid sequence as an important determining factor came to attention with the finding that mutations done in the regions responsible to form nuclei decrease the aggregation propensities, when the hydrophobicity is increased (or decreased) at the site of mutation (Chiti and Dobson 2006). Charge of a protein is another essential factor, as we all know that extreme high or low charge of a protein would impede its self-association. This is augmented by our understanding that interaction of a polypeptide with macromolecules having high compensatory charge increases their aggregation potential. Moreover, the tendency of a protein to attain beta-sheet conformation rather than alpha-helical structure is known to increase their chance of getting aggregated. It seems during the course of evolution, certain proteins have developed in such a way that their propensities to form beta sheet structure are avoided. It is assumed that because of these reasons most of the proteins acquire higher net charge and lower hydrophobicity than natively folded proteins to attenuate their aggregation tendencies in normal physiological conditions (Chiti and Dobson 2006; Uversky 2002).

8.4 Cellular Mechanisms of Toxicity

It is interesting that how the conduit from protein misfolding to proteopathy development follows almost similar chain of cytotoxic events, irrespective of the organ system affected. This section elaborates such toxicity causing mechanisms or events, such as functional disruption of cell organelles, protein quality control system and immune system, enhanced oxidative stress, generation of amyloid pores, etc.

8.4.1 Oxidative Stress and Mitochondrial Dysfunction

Mitochondria are ubiquitous intracellular organelles that perform various essential functions inside the cell such as ATP production, cellular calcium buffering, provision of free radicals, and apoptosis regulation. Its dysfunctioing due to the presence of protein aggregates can lead to number of deleterious consequences, such as impaired calcium balance, oxidative stress, and activation of the mitochondrial permeability transition pore (Shigenaga et al. 1994). Under oxidative stress, cellular ROS level elevates, attacking macromolecules like proteins, lipids, nucleic acids, etc. ROS-mediated lipid peroxidation is a known complication of pulmonary proteopathies, which imbalances phospholipid turnover and could rupture cell membranes. Moreover, abrupt oxidation of proteins via ROS could alter their structure so that either they become target for UPS mediated degradation or accumulate to form toxic aggregate species, thus perturbing physiological pathways and furthering cellular damage (Wilck and Ludwig 2014). Its abnormal functioning can also attenuate ATP production, causing reduced proteasome activity and enhanced retention of misfolded proteins in the cell, thereby strengthening the vicious circle of protein aggregates→mitochondrial dysfunction→oxidative stress→UPS failure→protein aggregates.

However, the mechanism of protein aggregation mediated PQC dysfunctioning is yet not clear and is a key area of interest, as it can provide with novel potential therapeutic targets. aSyn aggregates, most probably their protofibrils are believe to do so, by down regulation of complex I activity; mitochondrial fragmentation and excessive mitophagy. However, in the beginning, it was assumed that aSyn disrupts mitochondrial respiratory chain system, located between the outer and inner membranes, by binding with membrane phospholipids. But, a contemporary

study done on single SN neurons has indicated that there is no such direct association as they had observed increased levels of respiratory chain complex subunits within neurons containing aSyn pathology (Reeve et al. 2012). On top of that, a recent study done with mice expressing A53T aSyn variant revealed age-dependent changes in both mitochondrial morphology and proteins regulating mitochondrial fission and fusion, indicating aSyn to impair normal mitochondrial dynamics. Additionally, the existence of histological and morphological changes in mitochondria in normal myoblasts with transgenic APP gene had shown involvement of mitochondrial dysfunctioning in AD pathogenesis. Studies done with ALS patients, mice and cellular models have also reported enhanced calcium levels and reduced respiratory chain complexes I and IV activity in mitochondria (Wijesekera and Leigh 2009). Similar observations were reported in CFTR deficient cells, where elevated ROS levels altered ATP utilization rate, impaired calcium metabolism, and perturbed electron transport system (Velsor et al. 2006).

8.4.2 Cell Membrane Disruption/ Amyloid Pores

Membrane permeabilization is a common element of misfolded protein toxicity and is known to be caused via formation of annular pores in cell membranes by oligomeric species of various proteins such as Aβ, IAPP, and aSyn. This can further cause various deleterious effects in cell, for example, Ca^{2+} immobilization, ER induction, ROS generation and ultimately, cell death (Herczenik and Gebbink 2008). Apart from dopamine/aSyn adduct formation in the cell, another reason for dopaminergic cell death in case of PD is believed to be disruption of ion gradients maintaining neuronal homeostasis because of membrane permeabilization caused by oligomeric aSyn.

8.4.3 Immune Dysfunction

In addition to their direct toxic effects, misfolded proteins can overwhelm immune system and instigate autoimmune responses. In the brain of AD patients, neuroinflammatory responses are manifested as local stimulation of the complement system, acute-phase responses, enhanced C-reactive protein expression, and activation of inducible nitric oxide synthase and prostaglandin generating cyclooxygenase-2. It has been found that Aβ aggregates trigger microglial cell activation leading to release of a variety of proinflammatory mediators including, complement factors, cytokines, secretory products ROS, and nitric oxide, ultimately causing neuronal cell degeneration (Hickman and El Khoury 2013). Similar kind of chronic inflammation is also visible in most of the other protein misfolding diseases, including ALS, PD, prion diseases, atherosclerosis, cystic fibrosis, etc. However, in the case of atherosclerosis and diabetes mellitus, inflammatory processes are mediated by macrophages not microglial cells. Interestingly, mutant htt seems to have some yet unknown arsenal via which it could compromise immune responses, cause motility and migration deficits in immune cells, thereby making host system susceptible to immune deficiency disorders (Kwan et al. 2012).

8.4.4 Damaged Protein Quality Control System

Impairment of various components of PQC system due to the presence of protein aggregates is a common toxic insult reported in proteopathies. While healthy PQC system could eliminate toxic aggregates, perturbing its function is sufficient enough to compromise normal cellular homeostasis, thus making them an important target for therapeutic intervention. In cardiomyocytes, reduction of protein aggregation or chaperone overexpression has been reported to prevent proteasomal functional

insufficiency, indicating impairment of PQC system to be mediated by aggregates. Few studies have suggested aggregates of polyglutamine expanded proteins like ataxin 1 to derange UPS function (Duenas et al. 2006). aSyn linked toxicity is also seen to be mediated by modulation of PQC mechanisms. aSyn overexpression studies has shown its involvement in the retention of damaged mitochondria in the cytosol by enhancing insolubility of Parkin, an E3 ligase, which leads to aberrant accumulation of toxic proteins like aminoacyl-tRNA synthetase, the far upstream element binding protein 1 and Paris, deregulating mitobiogenesis and ROS metabolism (Rochet et al. 2012). Brain tissues in AD show decreased content of Beclin 1, a protein involved in the initiation of autophagy, thereby suggesting autophagic lysosomal system impairment. . This was supported by the finding that transgenic mice overexpressing APP due to a deletion of one Beclin-1 allele increases the rates of the formation of amyloid plaques and accumulation of morphologically abnormal lysosomes.

8.4.5 Co-aggregation

Most of the misfolded protein aggregates acquire a peculiar ability to interact with the Wt proteins and coaggregate them as well, leading to fatal insufficiency of that protein along with the enhancement of cellular aggregate burden. Similar mechanism is expended by mutated p53 protein aggregates to coaggregate with Wt protein in healthy cells and spread cancer (Forget et al. 2013). Studies done with polyQ htt protein aggregates suggest primary composition of aggregated peptide to be an essential factor for determining its coaggregating propensities (Bak and Milewski 2010). However, in some of the cases, aggregates have been observed to sequester not only Wt proteins but also other cellular proteins, as in the case of Li-fraumeni syndrome, where p53 aggregates have been observed to nonspecifically interact and coaggregate with other tumor suppressor proteins like p63 and p73 thereby elevating cytotoxicity (Xu et al. 2011). Similar observations were made in a recent finding, where artificial protein aggregates displayed higher tendencies to coaggregate with crucial proteins such as those involved in transcription and cytoskeleton stabilization, explaining the intensified toxic effect on several cellular mechanisms (Olzscha et al. 2011). It has been alluded that proteins usually coaggregate with oppositely charged proteins due to nonspecific hetero-interchain interactions (Trivedi et al. 1997). In physiological conditions, chaperones prevent such conspicuous interactions among proteins, and in diseased cells they bind with aggregates to shield other proteins (Doyle et al. 2013).

8.5 Therapeutic Advances

This part of the chapter overviews the current treatment strategies employed against proteopathies. The current advances, advantages, and disadvantages of those strategies have also been mentioned.

8.5.1 Immunotherapy

Active and passive immunizations are aimed at inhibiting generation of protein aggregation and removal of aggregates that are already formed. The concept of passive immunotherapy includes raising monoclonal antibodies against toxic proteins which can then be injected into patients to decrease their amyloid or aggregate burden. Formerly, the so-called “first-in-man” and “first-in-kind” clinical trial for the development of a PD vaccine, PD01A has been initiated. This vaccination aims to educate the immune system to generate antibodies directed against aSyn, and hence neutralize its toxic impact (PRNewswire 2012). However, for AD, several types of Aβ peptide immunotherapy are under investigation, including direct immunization with synthetic intact Aβ1-42, active immunization involving the administration of synthetic fragments of Aβ

peptide conjugated to a carrier protein and passive administration with monoclonal antibodies directed against Aβ peptide (Schenk 2002). The first clinical trial was done with anti-Aβ vaccine AN1792, but due to the development of meningoencephalitis in a small percentage of patients all study dosing was halted. Moreover, immunization with Aβ1-42 was efficient in clearing amyloid plaques in patients with AD, but prevented progressive neurodegeneration (Delrieu et al. 2012). Several monoclonal antibodies for AD had also been tested: solanezumab (LY-2062430), PF-04360365, bapineuzumab (AAB-001), R-1450 (RO-4909832), GSK-933776, and MABT-5102A (Mangialasche et al. 2010). Out of these, solanezumab seems to be very promising as it presents less CNS adverse events and is the first therapeutic drug to be evaluated in the anti-amyloid treatment in asymptomatic AD prevention clinical trial. Investigators are now planning to test an Aβ-clearing drug in older people thought to be in the presymptomatic stage of AD (Alzforum 2013). Anti-inflammatory therapy is also seen as a potential therapeutic intervention for cardiac amyloidosis. Patients with AL amyloidosis have been reported to survive longer after treatment with melphalan and prednisolone (Kyle et al. 1999). Prednisolone acts by reducing the transcription of inflammatory mediators, cycloxygenase 2, and several cytokines including TNFα, IL-1, and IFNγ, while melphalan is a protein synthesis inhibitor. Additionally, anti-TNFα agents have been reported to substantially suppress the production of serum amyloid A, the protein associated with AA amyloidosis (Banypersad et al. 2012).

Recently, a therapeutic monoclonal antibody that is reactive with all types of amyloid has lately been developed by targeting serum amyloid A protein (SAP) because it is a universal constituent of all amyloid deposits and an excellent immunogen (Bodin et al. 2010). To ensure that anti-SAP antibodies reach residual SAP in the amyloid deposits, circulating human SAP can be depleted by the bis-d-proline compound CPHPC. This novel combined therapy has shown encouraging results in mice models and is expected to be effective for all forms of human systemic and local amyloidosis.

8.5.2 Antioxidative Therapy

ROS are generally harmful, which makes antioxidant defenses essential and then promising therapeutic targets. These agents can be classified on the basis of their *modus operandi* – (a) compounds preventing free radical formation; (b) compounds neutralizing free radicals by chemically interacting with them; and (c) compounds enhancing the ROS secondary metabolites thereby limit the extent of cellular damage. Glutathione (GSH), a potent antioxidant belonging to the second category of antioxidant agents, has been shown to relieve oxidative stress in neuronal cells. Recently developed L-dopa-GSH co-drugs with blood-brain barrier-crossing properties have been reported to effectively prevent MAO-mediated metabolism of dopamine (Cacciatore et al. 2012). Furthermore, methionine sulfoxide reductase, an antioxidant repair enzyme, had also been shown to assist the inhibition of aSyn fibrillation, and neurotoxicity by repairing oxidatively damaged protein and by depleting ROS (Liu et al. 2008). Antioxidant therapy has also been tried to treat AD either by increasing the pool of endogenous antioxidants (e.g. vitamins, coenzyme Q10, or melatonin) or by the intake of dietary antioxidants, such as phenolic compounds of flavonoid or non-flavonoid type. However, in clinical trials, these agents had shown limited success may be because of poor distribution or in case of ND, agent's inherent difficulty to cross the brain–blood barrier. Shockingly, clinical trials to test the efficacy of antioxidants in treating diabetes patients reported contradictory results, describing elevated antioxidative stress agents to be negatively correlated with oxidative stress culmination (Golbidi et al. 2011). But, such results could be because of inadequate study design or selected targets. Further, clinical trial studies are required to clearly understand the importance of antioxidative therapy.

8.5.3 Modulating PQC Mechanisms

8.5.3.1 Ubiquitin Proteasome System

Most of the therapeutic strategies targeting UPS aim at enhancing their efficiency to remove mis-

folded proteins and their deposits from the cell. Following this stratagem, various modulators of UPS have been found and are being clinically tested. Genome-wide association studies have identified negative regulator of ubiquitin-like protein 1 (NUB1) as a modifier of mutant htt abundance. NUB1 overexpression has been reported to rescue neuronal cells from cell death by increasing proteasomal degradation of mutant htt, suggesting interferon-β (known inducer of NUB1) as a potential therapeutic agent in HD treatment (Lu et al. 2013). Similarly, upregulation of the 11S proteasome in mice models with desmin-related cardiomyopathy had been demonstrated to reduce aberrant protein aggregation and prevent cardiac dysfunction (Li et al. 2011). Contrastingly, bortezomib, a selective 26S proteasome inhibitor, has emerged as an effective drug against cancer and cardiac amyloidosis, and its use for treating multiple myeloma has been recently approved by US food and drug administration (Adams and Kauffman 2004). It seems that impairing proteasomal function enhances ER stress and encourages cell to undergo apoptotic cell death without affecting overall functioning of the organ system affected (Hedhli et al. 2008). Currently, for treatment of patients suffering from cardiac amyloidosis with bortezomib in conjugation with other agents is undergoing phase II and phase III clinical trials (Banypersad et al. 2012).

8.5.3.2 Molecular Chaperones

Upregulation of molecular chaperones is considered to inhibit aggregation by facilitating the refolding of misfolded proteins or by directing the aggregated proteins to cellular clearance pathways. Using various cellular models and *in vitro* studies, upregulation of heat shock proteins like Hsp70, Hsp40 and Hsp27 have been shown to attenuate aSyn fibril formation (Rochet et al. 2012). Also, Hsp70 overexpression is found to be an effective treatment in mouse model of SCA1 disease and fly model of HD (Chaudhuri and Paul 2006). Interestingly, intermediate concentrations of molecular chaperone Hsp104 in yeast models have been reported to enhance the propagation of yeast non-Mendelian factor [psi+], analogue of mammalian prions, while at higher concentrations, it cleared [psi+] from the cell. These results suggest modulating cellular levels of molecular chaperone as another potential therapeutic target (Chernoff et al. 1995).

8.5.3.3 Autophagy

Rapamycin is a potent autophagy inducer that works via inhibiting mTOR's (molecular target of rapamycin) kinase activity. Studies done with cellular models have shown rapamycin treatment to enhance degradation of toxic fibrillated proteins such as mutant ataxin, aSyn, htt, and tau (Hochfeld et al. 2013). Similar results were obtained with fly and mouse models of HD, where rapamycin effectively cleared mutant htt from the cell, reducing aggregate load and toxicity in the cell. This beneficiary was exclusively brought about by autophagy induction as this drug did not prove effective in the fly models of various proteopathies with reduced activity of autophagy genes (Wang et al. 2009). Rapamycin analogue CCI-779 had also been shown to reduce levels of toxin proteins in drosophila models of SCA3 and HD (Menzies et al. 2010; Ravikumar et al. 2004). Since mTOR is also involved in ribosome biogenesis and protein translation, inhibiting its activity for a long duration of time leads to deleterious side effects. Alternatively, various other autophagy inducers lacking these side effects have been introduced such as lithium, sodium valproate, carbamazepine, xestospongin B, and rilmenidine have also been introduced (Hochfeld et al. 2013). They have been shown to reduce toxicity and clear aggregates in fly models of HD, and rilmenidine being the drug with minimal side effects is currently under clinical trials with HD patients (Rose et al. 2010).

8.5.4 Gene Therapy

The main aim of gene therapy is to replace the mutant gene with the Wt copy wherever it is needed in the body. However, it is the delivery of gene to the target organ that makes it difficult for treating every genetic disease. The major breakthrough came in this regard with the development

of a modified virus carrying Wt CFTR gene to be delivered into the lungs of CF patients in the form of aerosols (Laube 2014). Other than viruses, liposomes have also emerged as an alternative vector to deliver Wt CFTR gene (Prickett and Jain 2013). The largest clinical trial of gene therapy for cystic fibrosis is currently being conducted by the UK Cystic Fibrosis Gene Therapy Consortium. It involves analyzing cystic fibrosis patients using an inhaler to breathe in a working copy of the cystic fibrosis gene once a month for a year. Other than CF, discoveries in this regard have been made to treat HD and PKU (Chowhan et al. 2013). Adeno-associated virus-mediated delivery of PAH gene for long-term improvement of PAH deficiency in PKU has been studied via mouse models, though the investigations have not advanced to human trials stage (Ding et al. 2005). However, the clinical trials for gene-therapy mediated treatment of HD have been started in 2014 by Roche and Isis Pharmaceuticals, Inc.

8.5.5 Reduction/Inhibition of Amyloid Formation

8.5.5.1 RNA Silencing or Antisense Therapy

The rationale for using antisense therapy against proteopathies is that the RNA interference (RNAi) mediated allele-specific silencing of the disease associated gene will repress gene expression of mutant protein and maintain heterozygous levels of Wt protein in the cell. This will eradicate the possibility of accumulation of misfolded proteins in the cell. Novel drugs aimed at utilizing RNA silencing and antisense oligonucleotide therapies for reducing production of TTR, protein associated with ATTR cardiac amyloidosis has been developed. ALN-TTR01, a systemically delivered RNAi therapeutic, is already in phase I clinical trial (Benson et al. 2006). In addition, the first clinical trial of intrathecal delivery of an antisense oligonucleotide, ISIS 333611, against mutant SOD1 messenger RNA has revealed this antisense therapy to be safe in ALS patients (Miller et al. 2013). It is believed that the gene silencing strategy cannot be utilized in diseases with defective PQC or where its efficiency has declined with age, such as in most cases of PD and AD (Gregersen 2006). But, recently a new specific SOFA-hepatitis delta virus ribozyme has been engineered that was exploited as RNA silencing tool against APP gene in AD cell models and demonstrated to be effective in decreasing variant APP mRNA and protein levels, as well as misfolded Aβ levels (Ben Aissa et al. 2012).

8.5.5.2 Stabilization of Fibril Precursor Protein

It is known that amyloid formation requires conversion of majority of alpha helices of respective precursor protein into β sheets to form fibril precursor protein which has an alternate aggregate-prone conformation that triggers aggregate accretion and propagation seeding reaction. It has been hypothesized that if we stabilize this soluble precursor protein by binding it to some pharmaceutical, then this process of amyloidosis will be inhibited. This has been lately explored in ATTR cardiac amyloidosis, where effects of drugs like diflunisal and tafamidis on TTR structure stability are going on (Banypersad et al. 2012). Higher affinity superstabilizers, palindromic ligands have also been developed that inhibit amyloid formation in ATTR cardiac amyloidosis through their protein stabilization effect (Kolstoe et al. 2010). Additionally, small peptides are being synthesized that can bind specifically with the fibril precursor protein to block and/or reverse its abnormal conformational change (Estrada and Soto 2006).

Alternatively, extensive investigations have led to the finding of small compounds, such as indomethacin, Congo red, 2,4-dinitrophenol, cyclodextrin derivatives, curcumin, meclocycline, di- and tri-substituted aromatic molecules, and hematin, which can inhibit protein aggregation by stabilizing native conformation of the aggregation prone protein to inhibit their oligomerization (Miroy et al. 1996; Necula et al. 2007).

8.5.5.3 Modulation of Cellular Enzymes

A recent finding of a specific mutation in the gene encoding for APP that can significantly decrease its cleavage by beta-secretase and confer resistance to the development of AD in patients has reinforced the hypothesis that the inhibition of Aβ cleaving secretases is an important disease modifying approach (Jonsson et al. 2012). Following this trend, Merck USA has started largest phase II/II clinical trial of MK8931, a BACE-1 (β-secretase APP cleaving enzyme) inhibitor in AD (Merck&Co. 2013), instead of the failure of phase 3 trial in 2010 of a gamma-secretase inhibitor drug, semagacestat in prodromal AD because of its adverse effects seen on worsening of cognition (Mckee 2010). Additionally, anticholinesterase inhibitors are currently approved by FDA for AD treatment. They are believed to work either by rescuing AD brains from acetylcholine deficiency or by preventing abrupt neuronal stimulation by antagonizing NMDA-type glutamate receptors (Chen et al. 2014a). Since hyperphosphorylated tau are present in the neurofibrillary tangles in brains of AD patients, inhibitors of tau kinases are also considered an important therapeutic targets, but it is still a relatively unexplored avenue (Huang and Mucke 2012). Recently, enhanced GCase levels have been reported to revert aSyn related pathophysiological attributes and advance cognition in mouse models of gaucher's disease. It has been hypothesized that correcting GCase deficiency could affect SNCA gene expression and prevent PD development (K Chowhan et al. 2014; Schapira and Gegg 2013). Utilizing cellular enzymes as therapeutic targets have also been experimented for treating PKU, where the introduction of L-Phe metabolizing enzyme, phenylalanine ammonia lyase, in intestinal lumen was observed to efficiently metabolize and digest L-Phe (Harding 2008).

8.5.5.4 Tetracyclines

The tetracyclines are a family of broad spectrum antibiotics that include tetracycline, doxycyclin, and minocycline. Studies done with transgenic mice models have revealed tetracycline to inhibit IAPP amyloid formation by hampering misfolding of soluble oligomers, and hence delay development of type-2 diabetes (Aitken et al. 2010). Since they can cross blood–brain barrier, they have been utilized in neurodegenerative diseases also. Minocycline has been shown to prevent Aβ and tau protein accumulation in AD models (Noble et al. 2009), while tetracycline was observed to inhibit the conversion of prion protein, PrP^{C} into PrP^{Sc} form and hinder prion aggregation (Tagliavini et al. 2000). Furthermore, doxycycline displayed prolonged survival and delayed onset of disease in animal models of prions (Forloni et al. 2002).

8.5.5.5 Osmolytes

Osmolytes or chemical chaperones are small organic compounds known to have the capability to correct the altered structure of misfolded proteins by influencing the rate or fidelity of their folding reaction, thereby preventing UPS degradation mediated cellular deficiency of that protein. They have been reported to converse the intracellular retention of several different misfolded proteins such as prion protein, α-galactosidase A, aquaporin-2, CFTR, vasopressin V2 receptor, p53, AAT, and P-glycoprotein (Chaudhuri and Paul 2006). Osmolytes such as glycerol and 4-phenylbutyric acid have been reported to increase the secretion efficiency of Wt as well as variant AAT in cell lines and transgenic animals. In CF, detrimental effects of ΔF508 mutation can be reverted by treating cells with osmolytes like glycerol, dimethyl sulfoxide (DMSO), trimethylamine-N-oxide (TMAO), and deuterated water (Brown et al. 1996). However, effects of some osmolytes like TMAO on protein aggregation are concentration dependent. TMAO at higher concentration (>3 M) leads to tight folding of aSyn eventually forming stable oligomers and decreased fibrillation rate, but at low concentrations it forms partially folded intermediate which accelerates fibrillation (Fink 2006). Because of these complex effects of osmolytes on aggregation kinetics, utilizing it for treatment will require very stringent investigations. Additionally, target-based virtual screening has shown pharmacological chaperones as potential candidates to treat PKU

via stabilizing PAH and preventing its stability (Harris 2014).

8.5.5.6 Polyamines

Effects of various naturally occurring cosolutes like polyamines on protein aggregation are extensively investigated in order to find a therapeutically important molecule. A recent gene expression analysis done on differentially affected brainstem regions of PD patients had implicated higher polyamine levels to be one of the causes for induction of aSyn aggregation (Lewandowski et al. 2010). Furthermore, it has been found that the pathological length polyQ proteins like mutated htt induce polyamine synthetic pathway, which further enhance their aggregation and trigger apoptotic cell death (Chowhan and Singh 2012). Thus, indicating reduction of polyamine levels as a potential therapeutic target for PD and HD.

8.6 Conclusions and Future Perspectives

Protein misfolding is indeed a major etiology for several localized and systemic diseases. Genetic mutations, cellular stress, failed unfolded protein response, and overwhelmed protein folding and trafficking system all together or independently instigate accumulation of misfolded protein aggregates and cause cellular deficiency of its native counterpart. This directly influences the proper functioning of cellular mechanisms involving the misfolded protein in addition to the cytotoxicity caused by its oligomers and amyloid fibrils. Plenty of resources have been expended up till now to find cure for these diseases but what we have achieved so far are more or less symptomatic treatments, nothing providing permanent relief. The novel therapeutic strategies like gene therapy, peptidomimetics, protein stabilizing agents like osmolytes and polyamines, etc. though seem promising have yet to prove their competence in clinical trials. We believe that in addition to counteracting the toxic effects of protein oligomers and amyloid fibrils, developing novel strategies focused on targeting the primary cause of proteopathies, i.e. protein misfolding, will prove to be a universal remedy for all protein misfolding-related disorders. This could be done either by developing agents that could augment correct folding of mutated proteins or by employing tactics to decrease the load of toxic protein oligomers and fibrils. Furthermore, it has to be understood that to discover new treatment approaches, we have to unravel the entire physiological phenomenon involved with a disease, and to gain all this information we require strenuous mechanistic investigations.

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Pharmacological Chaperones in Protein Aggregation Disorders

9

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Contents

Abstract

Chaperones are molecules that assist in the proper folding of nascent polypeptide chain and prevent protein from aggregation. There are at least three types of chaperones: molecular chaperones, chemical chaperones and pharmacological chaperones. Unlike molecular chaperones that are enzymes or proteins, chemical chaperones are small molecular weight organic compounds. Molecular chaperones catalyze a folding process, while chemical chaperones simply assist in the protein folding process by making the malfolded or unstable protein stay away from aggregation. On the other hand, pharmacological chaperones are a class of molecules that bind and inhibit the unstable or misfolded proteins. Therefore, they act specific for a particular protein. Various studies have yielded insights that these pharmacological chaperones would be effective for a large number of diseases including Alzheimer's disease, nephrogenic diabetes insipidus, cystic fibrosis, lysosomal storage diseases, phenylketonuria, methylmalonic aciduria, etc. In the present chapter, we have described various diseases for which the use of pharmacological chaperones has been proved successful.

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Pharmacological chaperones • Protein misfolding • Lysosomal storage diseases • Phenylketonuria • Methylmalonic aciduria

9.1 Introduction

Protein misfolding and aggregation is one of the vexing issues in protein biophysics and is the primary cause of several human diseases. Indeed, the production of misfolded proteins due to defects in the protein folding pathway is the main cause of such diseases. These misfolded proteins if not degraded by the proteosome or taken care of by the protein quality control system cause retention in the intracellular environment or in the endoplasmic reticulum. It has been known that certain small molecule compounds including chemical chaperones and certain manipulation of the molecular chaperone network help to clear the protein retention and bring back the misfolded protein to the folded native conformations. Nowadays, another class of chaperone called pharmacological chaperone has been shown to alleviate the mutant protein functional deficiency by binding to the misfolded protein and reverting the misfolding process. Pharmacological chaperones are an innovative class of small cell-permeable molecules that act by stabilizing unstable or misfolded proteins. The pharmacological chaperone strategy has been a promising approach for a large number of human diseases associated with protein conformational diseases. Furthermore, pharmacological chaperones have been shown to play a role in the inhibition of aggregation of unstable proteins due to its effect to stabilize native proteins and, therefore, are of great importance for the treatment of amyloidosis. Obvious examples are cystic fibrosis, amyloidosis, Parkinson's disease, Alzheimer's disease and Lou Gehrig's disease. Importantly, most metabolic disorders are caused due to mutations in the underlying gene that in fact disrupts the native protein structure and destabilizes it. It is commonly believed that mutations do not in majority of the cases target the key catalytic residues rather affect residues outside the catalytic core of the protein molecule. This results in the production of misfolded or largely unstable protein conformation that undergoes either enhanced proteosomal mediated degradation or aggregated inside the cells. In some cases, the misfolded proteins fail to exit from the ER. Enzyme-replacement therapy has been one of the currently undergoing practices against these proteopathic disorders. However, the treatment approach is highly expensive and quite often the injected enzyme fails to target the desired malfunctioned organs. Pharmacological chaperones are small molecules and therefore can be given orally, inexpensive and work specifically against the misfolded protein at the specific target tissue of the body (Sawkar et al. 2006). It is also known that these pharmacological chaperones also have the ability to cross the blood–brain barrier and thus are agents for the treatment of neurodegenerative diseases. Therefore, using pharmacological chaperones for the treatment of misfolding diseases might serve as an important therapeutic strategy because of their ability to stabilize the unstable proteins irrespective of their location. Interestingly, it has been shown that nearly all of the pharmacological chaperones now under development are active site ligands. However, tight specific binding anywhere on the surface of a protein ought to confer stabilization as a result of the increased number of interactions. In this context, for many years, it was thought that essentially the whole surface of a protein should be available for small molecule binding – that is, that proteins are sticky everywhere. But in the early 1990s, one of us established that only a small number of sites were actually available to bind organic molecules, because tightly bound water prevents access to much of the protein surface (Ringe 1995). These sites can be mapped crystallographically (Mattos et al. 2006) and computationally (Landon et al. 2009). Recently, these methods have been applied to a few enzymes, such as β-glucocerebrosidase (GCase), for the purpose of identifying sites other than the active site, known as exosites, that are suitable for pharmacological chaperone binding. For GCase, a single exosite was identified and a

library of small organic compounds (chemical libraries) has been docked to that position on the protein surface. It was found that binding of PC at the exosite of GCase was able to increase its thermal stability. However, for most of the diseases, such kinds of studies have still not started and are in infancy. Because of the reasons that identifying a protein's potential "docking sites," to which chaperones can bind, requires prior knowledge of 3D structure of those proteins. To discover pharmacological chaperones for specific targets is challenging for several reasons, including the characteristics of the protein surfaces, the lack of suitable chemical libraries and the shortage of efficient high-throughput screening (HTS) methods. However if not at large scale, but some instances whether a small molecule will act as a pharmacological chaperone or not can be inferred (Lieberman et al. 2007) by high-throughput screening of a compound library which can give greater flexibility and promise for the treatment of many untreatable dreaded diseases. In this chapter, we have tried to review some of the pharmacological chaperones for their use as therapeutic agents in protein aggregation diseases.

9.2 Pharmacological Chaperones in Alzheimer's Disease (AD)

Retromer is a multiprotein complex that is responsible for sorting and trafficking of cargo from endosomal vesicles to the trans side of Golgi complex or to the surface of cell (Cullen and Korswagen 2012; Seaman 2012). Retromer-mediated transport was first identified in yeast (Seaman et al. 1997, 1998). The complex consists of a core made up of a trimeric 'vacuolar protein sorting' (VPS) proteins (Vps35–Vps29–Vps26). The trimeric core of the complex binds to a sorting protein nexin (SNX) (Seaman 2005). Studies in mammalian cells discovered that the trimeric core of the complex is highly conserved, while its binding partner SNXs has been found to be highly variable. Recently, it has been shown that the mammalian Vps35–Vps29–Vps26 core besides forming a complex with Snx1–Snx2, Snx5–Snx6 and Snx3 can also bind with Snx27 (Cullen and Korswagen 2012; Seaman 2012). Besides binding the SNXs, it has been reported that the mammalian retromer complex can bind to other proteins/protein complexes, like Wiskott-Aldrich protein and SCAR homolog (WASH) complex (Burd and Cullen 2014). Binding to such complexes have been observed to modify its defined function of trafficking the cargo out of the endosomes. Dysregulation of retromer-mediated endosomal protein sorting was found in several neurodegenerative diseases such as Alzheimer disease (Small et al. 2005). Several cell culture studies and animal models (Lane et al. 2010; Wen et al. 2011; Small et al. 2005; Muhammad et al. 2008) have established that deficiencies in Vps26 and Vps35 were found to be responsible for increase in the levels of Aβ and other APP (amyloid-precursor protein) fragments (the main cause of AD) as revealed by cell culture and animal model studies (Fjorback et al. 2013; Nielsen et al. 2007). Normally, the neuronal retromer protein complex protects the cleavage of APP in endosomes, a process which is opposite in case of AD (Vardarajan et al. 2012). Thus specially designed molecules that can oppose the mutational deficiency of retromer would be of great use for the treatment of AD.

In this context, Maccozzi et al. used pharmacological chaperone for the first time to elevate the levels of retromer and enhance the stability of the retromer-APP complex (Mecozzi et al. 2014). They identified a pharmacological chaperone that can bolster entire core structure of the retromer complex so that it becomes less prone to degradation. Using the crystal structure of the retromer protein, the chaperone binding site was computationally evaluated that can enhance retromer stability. For that matter, the authors carried out *in silico* screening of large amount of small molecule compounds that can act alleviate retromer stability and were able to identify one effective pharmacological chaperone, R55. They found that R55 can enhance the stability of the retromer complex by 10 °C and increase the level of retromer in hippocampal neuronal cell cultures. It was also observed that in the presence of R55 the levels of APP in endosomes were lowered.

9.3 Pharmacological Chaperones in Nephrogenic Diabetes Insipidus

Nephrogenic diabetes insipidus (NDI) is an acquired or inherited disorder in which the nephritic system is not able to concentrate urine in response to arginine vasopressin (AVP) hormone. It has been known that mutations in the AVPR2 gene and AQP2 gene is the main underline cause of the disease (Langley et al. 1991; Deen et al. 2000). Mutations in AQP2 gene and AVPR2 were found to be responsible for around 10 % and 90 % of NDI, respectively. Out of the 150 mutations known in AVPR2, majority of the mutations cause protein misfolding leading to its retention in endoplasmic reticulum (ER) (Robben et al. 2005). Similarly, most of the mutations associated with AQP2 are confined to the six transmembrane domains, C− terminal domain and five connecting loops of AQP2. These three domains form the main core region of the protein. Due to these mutations, the protein misfolds and therefore are retained in the ER, which then is directed for ERAD (Endoplasmic reticulum associated degradation) mediated degradation (Deen et al. 1995; Mulders et al. 1998; Marr et al. 2001; Lin et al. 2002; Marr et al. 2002). It has been reported earlier that seven mutations of AVPR2 can be corrected by the use of pharmacological chaperones, SR121463A and VPA-985, which are in fact nonpeptide antagonists of the AVPR2 protein (Morello et al. 2000). These pharmacological chaperones, SR121463A and VPA-985, were also found to be permeable to the cell membrane and are able to target the AVPR2 to the cell surface. Using immunofluorescence experiments, the authors besides observing cellular localization were able to detect accumulation of vasopressin dependent AMP in presence of the pharmacological chaperone. In another development, pharmacological chaperone (SR49059) was also found to correct the functional deficiency in arginine vasopressin receptor 1a (AVPR1a) (Bernier et al. 2006), leading decrease water uptake and urine volume by 30 %.

9.4 Pharmacological Chaperones in Cystic Fibrosis

Cystic fibrosis transmembrane conductance regulator (CFTR) is basically a chloride ion channel of 180 kDa. It is present in the plasma membrane of epithelial cells, mainly the pulmonary system (Riordan 2008). Mutations in the CFTR gene have been found to be responsible for cystic fibrosis and are mainly confined to Caucasian population. Out of 1,200 mutations reported in CFTR, over 90 % are deletion mutations which are mainly confined to the Phenylalanine residue 508 and therefore are commonly referred to as ΔF508 (Kartner et al. 1991). The deletion mutation consequently leads to the loss of functional ability of CFTR. CFTR being synthesized on rough endoplasmic reticulum undergoes co-translational translocation in the ER, where in its further folding and other posttranslational modifications particularly glycosylation takes place (Kopito 1999). Further folding and processing i.e. glycosylation of CFTR takes place in golgi complex. It is a well-known fact that the extent of glycosylation differentiates ER proteins from the golgi ones. Golgi complex in turn targets the CFTR to the cell membrane where it acts as a chloride ion channel. ΔF508 CFTR is considered to be misfolded and therefore undergoes enhanced degradation by the ubiquitin-proteosome system. In another development, ΔF508CFTR is also demonstrated to be retained in ER that undergoes ERAD mediated degradation as evident from the fact that mutated CFTR proteins had not undergone complex glycosylation (Kartner et al. 1992; Kopito 1999). Studies have earlier shown that pharmacological chaperones like quinazoline derivatives could reverse the misfolding and the trafficking defect of the ΔF508 mutant protein and thus restore transmembrane conductance (Wang et al. 2008) *in vitro*. The results indicate that quinazoline derivatives would be used for the therapeutic intervention of cystic fibrosis patients. Additionally, in cell culture models, it has been shown that 8-cyclopentenyl-1,3-dipropylxanthine increases the expression of ΔF508-CFTR and

hence the chloride currents. Furthermore, ΔF508-CFTR in 8-cyclopentenyl-1,3-dipropylxanthine treated cells were also found to have undergone proper membrane localization and complex glycosylation (Guay-Broder et al. 1995; Srivastava et al. 1999; Eidelman et al. 2001).

9.5 Pharmacological Chaperones in Lysosomal Storage Diseases

Lysosomal storage diseases are metabolic disorders which are caused due to mutations in the metabolic enzymes resulting in impaired intracellular turnover and disposal of a variety of substrates, including sphingolipids, glycosaminoglycans, glycoproteins and glycogen. The sequence of events for the lysosomal accumulation of these substrates have not been properly known but is believed to be due to cell and tissue damage because of some secondary biochemical and cellular events (Futerman and van Meer 2004; Ballabio and Gieselmann 2009). Over 50 such diseases have been discovered so far. Mutations in lysosomal storage disorders include missense, frame shifts, insertions, deletions and truncation due to stop codons. These mutations are known to convert its normal enzyme to its functionally deficient one. Therapeutic approaches for the treatment of lysosomal storage diseases include substrate reduction therapy and enzyme replacement therapy. The limitations include inadequate bio-distribution and expensiveness of the enzymes. Nowadays, use of pharmacological chaperone for the treatment of lysosomal storage diseases has been one of the best approaches. Effective pharmacological chaperone for Fabry, Gaucher and Pompe disease have already been available. Fan et al. (1999) demonstrated for the first time a chaperone effect of an imino sugar in lymphoblasts from patients with Fabry disease caused by the deficiency of the lysosomal hydrolase α-galactosidase A (GLA) that results in vascular endothelial damage, peripheral neuropathy, cardiac, renal and eye disease. It has also been shown that iminosugars, acetamidodeoxynojirimycin, acetamidodideoxynojirimycin, N-acetylglucosamine thiazoline and a related acetamido analogue of castanospermine can also be successfully used for the therapeutic intervention of Tay-Sachs disease, which is due to mutation in β-hexosaminidase (Tropak et al. 2004). Subsequently, other studies provided the proof of principle for the use of pharmacological chaperone for Gaucher disease (Yu et al. 2007), gangliosidosis G_{M1} (Matsuda et al. 2003) and G_{M2} (Tropak et al. 2004; Maegawa et al. 2007) and Pompe disease (Okumiya et al. 2007; Parenti et al. 2007).

9.6 Pharmacological Chaperones in Phenylketonuria

Phenylketonuria (PKU), characterized by hyperphenylalaninemia, is an inborn metabolic error due to mutations in phenylalanine-hydroxylase (PAH) gene. This enzyme in presence of (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin and molecular dioxygen as co-substrates catalyzes the hydroxylation of l-Phe to l-Tyr. More than 500 mutations of PAH have been reported in humans which are mainly responsible for mental retardation in PKU patients. The treatment strategy includes enzyme replacement therapy, gene therapy and restriction of dietary Phe (which is often considered to be expensive). In addition to this, pharmacological chaperones have now proved to be successful for the treatment of PKU. Tetrahydrobiopterin (BH_4), cofactor of PAH, has been reported to act as pharmacological chaperone that rescues enzyme function. Binding of BH_4 offers stabilization of the mutant PAH enzyme in BH_4-responsive PKU genotypes (Gersting et al. 2010; Erlandsen et al. 2004; Aguado et al. 2006). Though BH_4 stabilized wild-type PAH, enzyme got inactive from its native activated labile conformation (Shiman et al. 1990). It has been earlier shown that the inhibitory effect of BH_4 is due to the interaction with the regulatory domain of PAH. BH_4 exhibits less effectiveness in some PKU genotypes from the

fact that a very few BH_4 analogues are able to show chaperone effect (Phillips and Kaufman 1984; Kappock and Caradonna 1996; Erlandsen et al. 2003; Solstad et al. 2003). Based on the high throughput screening, Martinez and his co-investigators were able to discover two potential compounds that were able to increase activity levels of WT- and the mutant- PAH. The compounds are 3-amino-2-benzyl-7-nitro-4-(2-quinolyl)-1,2-dihydroisoquinolin-1-one and 5,6-dimethyl-3-(4-methyl-2-pyridinyl)-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one.

In similar kind of screening, Santos-Sierra et al. (2012) identified 84 candidate compounds with an affinity to bind to the active site of PAH. Out of the 84 candidate compounds, only six were found to increase enzyme activity and protein stability in cell-based assays against proteolytic degradation. Furthermore, two compounds, benzylhydantoin and 6-amino-5-(benzylamino)-uracil, were found to increase the Phe oxidation and enhance its concentration in the blood of PKU mice with benzylhydantoin more effective than that of tetrahydrobiopterin (Santos-Sierra et al. 2012).

9.7 Pharmacological Chaperones in Methylmalonic Aciduria

MMAB gene encodes for the protein, ATP: cobalamine (I) alaminadenosyltransferase (ATR) that catalyzes the conversion of vitamin B_{12} into adenosylcobalamin, a vitamin B_{12}-containing coenzyme for methylmalonyl-CoA mutase. Mutations in the gene are the primary cause of vitamin B_{12}-dependent methylmalonic aciduria (MA). Methylmalonic aciduria is a hereditary disease in which the body fails to metabolize certain fats (lipids) and proteins properly. The effects of MA pop up during the early infancy that may vary from mild to severe. Common symptoms in the affected infants are vomiting, dehydration, hypotonia, developmental delay, lethargy, an enlarged liver and failure to gain weight and grow at the expected rate. Symptoms with long-term complications include problem of feeding, intellectual disability, chronic kidney disease, inflammation of the pancreas, etc. Without treatment, MA patients can lead to coma and death in some cases. To date, there is no proper cure for MA. However, the current treatment strategy basically aimed at reducing the production of methylmalonic acid by dietary limitation of the substrate. Recently, it has been shown that the pharmacological cobalamin can be very effective against MA by allowing the mutated ATR to function that shows clinical response in less than 40 % of MA patients. However, in many cases mental problem persists. Although it is difficult to normalize the level of the toxic metabolite, cobalamin supplementation shows great improvements in clinical condition, metabolic variables and growth (Fowler et al. 2008). In a very promising study, more than 2,000 different compounds were screened for the possible pharmacological chaperonic effect on the mutant ATR. It was found that six chemical analogs have the capability to act as chaperone for the mutant ATR. Furthermore, the authors investigated the effect of the six pharmacological chaperones on the stability of mutant and wild type ATR expressed in bacteria. It was observed that all the compounds enhance the stability of the mutant and the wild type ATR. Interestingly, the compound V (*N*-{[(4-chlorophenyl) carbamothioyl] amino}-2-phenylacetamide) significantly enhances the mutant protein stability and does not have any inhibitory effect on the purified wild type protein. Importantly, compound V has been shown to increase the ATR activity in fibroblasts cells derived from patients that harbours the mutant ATR protein (Isoleucine to Threonine substitution at position 96) in a hemizygous state to within control range. Interestingly, co-administration of the compound V with cobalamin was also observed to further increase the activity of the mutant ATR function. Additionally, the compound V was again examined for its efficacy on the animal models (by oral administration to C57BL/6J mice). Analysis of the steady state level of the ATR from the disease relevant organs (liver and brain) for 12 days revealed that

the ATR levels were significantly increased. Taken together, the results gave a clear picture that pharmacological chaperones can by employed for the treatment of MA patients. It is now important to screen for patients responsive against the compound V.

9.8 Pharmacological Chaperones in Retinitis Pigmentosa

Retinitis pigmentosa is caused by mutations in rhodopsin. Up to 150 different mutations have been identified to lead to retinitis pigmentosa with most of the mutations being missense in nature (Farrar et al. 2002; Stojanovic and Hwa 2002). New mutations leading to retinitis pigmentosa continue to appear (Schuster et al. 2005). Extensive biophysical investigations have been done so far in an attempt to trace the molecular defects of the several rhodopsin mutation associated with retinitis pigmentosa (Sung et al. 1991; Kaushal and Khorana 1994). The first mutation identified to lead to retinitis pigmentosa was P23H (Dryja et al. 1990). This mutant is retained in the ER in misfolded conformation and is prone to aggregation.

To improve the folding of rhodopsin mutants, retinoids have been used as potential pharmacological chaperones. For instance, folding efficiency of mutants has been observed to increase in the presence of 11-*cis*-retinal, 9-*cis*-retinal and 11-*cis*-7-ring retinal in the culture media demonstrated that these can be used as potential therapeutic agents to reduce the burden of such aggregation diseases (Saliba et al. 2002). In fact, vitamin A supplementation in diet reduces retinal degeneration as a result of rhodopsin aggregates accumulation (Li et al. 1998).

9.9 Pharmacological Chaperones in Prion Disease

Prion diseases are neurodegenerative protein-misfolding disorders which involve misfolding of the cellular prion protein (PrP^C). PrP is known to have a structured region and an unstructured region. Residues 124–231 encode the structured region of PrP and contain three alpha-helices and a small two-strand antiparallel beta-sheet (Riek et al. 1996; Zahn et al. 2000). A large unstructured region is at the N terminus of PrP and three unstructured residues are at the C terminus. Numerous ligands have been reported to bind to PrP^C. It has been suggested that ligands that can bind to the structured domain could potentially act as pharmacological chaperones. For instance, the cationic porphyrin, Fe(III)-TMPyP [Fe(III) meso-tetra (N-methyl-4-pyridyl)porphyrine] was observed to bind to the structured domain leading to stabilization of PrP^C thereby acting as a pharmacological chaperone (Nicoll et al. 2010). Furthermore, Fe(III)-TMPyP has been reported to have good anti-prion activity in cell culture (Caughey et al. 1998) and animal models (Priola et al. 2000).

9.10 Conclusion and Future Perspectives

Increasing number of studies have so far shown that small molecules like chemical chaperones and pharmacological chaperones can facilitate protein folding and their processing which makes them a potential therapeutic agents for various protein misfolding diseases. Among these two, pharmacological chaperones are very selective in binding and stabilizing proteins and promise good avenues for the development of clinically useful therapeutic agents. However being specific, the use of pharmacological chaperones has restricted their use as prior knowledge or information of the target protein is important. So it becomes very important in developing such pharmacological chaperone molecules which can be active for larger subset of diseases.

Although pharmacological chaperones have been clinically tested but very few limited trials have been done. So in future, more exhaustive screening along with clinical trials aiming at identifying clinically potent pharmacological chaperones need to be carried out.

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