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1.1 INTRODUCTION

This chapter is dedicated to the description of the intrinsically disordered chaperones and their roles in neurodegenerative diseases. Three major concepts, namely, intrinsically disordered proteins (IDPs), chaperones, and neurodegeneration are briefly introduced below.

1.1.1 Intrinsically Disordered Proteins

1.1.1.1 Concept. Evidence is rapidly accumulating that many protein regions and even entire proteins lack stable tertiary and/or secondary structure in solution, existing instead as dynamic ensembles of interconverting structures. These naturally flexible proteins are known by different names, including intrinsically disordered (Dunker *et al.*, 2001), natively denatured (Schweers

Protein Chaperones and Protection from Neurodegenerative Diseases, First Edition. Edited by Stephan N. Witt.

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et al., 1994), natively unfolded (Uversky et al., 2000; Weinreb et al., 1996), intrinsically unstructured (Tompa, 2002; Wright and Dyson, 1999), and natively disordered proteins (Daughdrill et al., 2005). By "intrinsic disorder," it is meant that the protein exists as a structural ensemble, either at the secondary or at the tertiary level. In other words, in contrast to ordered proteins whose 3D structure is relatively stable and Ramachandran angles vary slightly around their equilibrium positions with occasional cooperative conformational switches, IDPs or intrinsically disordered regions (IDRs) exist as dynamic ensembles in which the atomic positions and backbone Ramachandran angles vary significantly over time with no specific equilibrium values and typically undergo noncooperative conformational changes. To some extent, conformational behavior and structural features of IDPs and IDRs resemble those of nonnative states of "normal" globular proteins, which may exist in at least four different conformations: ordered, molten globule, premolten globule, and coil-like (Fink, 2005; Uversky, 2003b; Uversky and Ptitsyn, 1994, 1996b). Using this analogy, IDPs and IDRs might contain collapsed disorder (i.e., where intrinsic disorder is present in a molten globular form) and extended disorder (i.e., regions where intrinsic disorder is present in the form of a random coil or premolten globule) under physiological conditions in vitro (Daughdrill et al., 2005; Dunker et al., 2001; Uversky, 2003b).

1.1.1.2 Experimental Techniques for IDP Detection. The disorder in IDPs has been detected by several physicochemical methods elaborated to characterize protein self-organization. The list includes, but is not limited to, X-ray crystallography (Ringe and Petsko, 1986), NMR (nuclear magnetic resonance) spectroscopy (Bracken et al., 2004; Daughdrill et al., 2005; Dyson and Wright, 2002, 2004, 2005a, b), near-UV circular dichroism (CD) (Fasman, 1996), far-UV CD (Adler et al., 1973; Provencher and Glockner, 1981; Uversky et al., 2000; Woody, 1995), optical rotatory dispersion (ORD) (Adler et al., 1973; Uversky et al., 2000), FTIR (Fourier transform infrared spectroscopy)(Uversky et al., 2000), Raman spectroscopy and Raman optical activity (Smyth et al., 2001), different fluorescence techniques (Receveur-Brechot et al., 2006; Uversky, 1999), numerous hydrodynamic techniques (including gel filtration, viscometry, small angle X-ray scattering (SAXS), small angle neutron scattering (SANS), sedimentation, and dynamic and static light scattering) (Receveur-Brechot et al., 2006; Uversky, 1999), rate of proteolytic degradation (Fontana et al., 1997, 2004; Hubbard et al., 1994; Markus, 1965; Mikhalyi, 1978), aberrant mobility in sodium dodecyl sulfate (SDS)-gel electrophoresis (Iakoucheva et al., 2001; Tompa, 2002), low conformational stability (Privalov, 1979; Ptitsyn, 1995; Ptitsyn and Uversky, 1994; Uversky, 1999; Uversky and Ptitsyn, 1996a), H/D exchange (Receveur-Brechot et al., 2006), immunochemical methods (Berzofsky, 1985; Westhof et al., 1984), interaction with molecular chaperones (Uversky, 1999), electron microscopy or atomic force microscopy (Miyagi et al., 2008; Receveur-Brechot et al., 2006), and the charge state analysis of electrospray ionization mass spectrometry (Frimpong et al., 2010; Kaltashov

and Mohimen, 2005). For more detailed reviews on methods used to detect intrinsic disorder, see Bracken *et al.* (2004), Daughdrill *et al.* (2005), Longhi and Uversky (2010), Receveur-Brechot *et al.* (2006), and Uversky (2002a).

1.1.1.3 Sequence Peculiarities of IDPs and Predictors of Intrinsic Disorder. IDPs and IDRs differ from structured globular proteins and domains with regard to many attributes, including amino acid composition, sequence complexity, hydrophobicity, charge, flexibility, and type and rate of amino acid substitutions over evolutionary time. For example, IDPs are significantly depleted in a number of so-called order-promoting residues, including bulky hydrophobic (I, L, and V) and aromatic amino acid residues (W, F, and Y), which would normally form the hydrophobic core of a folded globular protein, and also possess low content of C and N residues. On the other hand, IDPs were shown to be substantially enriched in the so-called disorder-promoting amino acids: A, R, G, Q, S, P, E, and K (Dunker et al., 2001; Radivojac et al., 2007; Romero et al., 2001; Williams et al., 2001). Many of the differences mentioned were utilized to develop numerous disorder predictors, including PONDR® (Li et al., 1999; Romero et al., 2001), CH-plot (Uversky et al., 2000), NORSp (Liu and Rost, 2003), GlobPlot (Linding et al., 2003a, b), FoldIndex© (Prilusky et al., 2005), IUPred (Dosztanyi et al., 2005), and DisoPred (Jones and Ward, 2003; Ward et al., 2004a, b) to name a few. It is important to remember that comparing several predictors on an individual protein of interest or on a protein data set can provide additional insight regarding the predicted disorder if any exists.

1.1.1.4 Natural Abundance of IDPs and Their Biological Functions. Application of various disorder predictors to different proteomes revealed that intrinsic disorder is highly abundant in nature and the overall amount of disorder in proteins increases from bacteria to archaea to eukaryota, with over a half of the eukaryotic proteins containing long predicted IDRs (Dunker *et al.*, 2000; Oldfield *et al.*, 2005; Ward *et al.*, 2004b). One explanation for this trend is a change in the cellular requirements for certain protein functions, particularly cellular signaling. In support of this hypothesis, an analysis of a eukaryotic signal protein database indicated that the majority of known signal transduction proteins were predicted to contain significant regions of disorder (Dunker *et al.*, 2002a).

Although IDPs fail to form unique 3D structures under physiological conditions, they are known to carry out a great number of important biological functions, a fact that was recently confirmed by several comprehensive studies (Daughdrill *et al.*, 2005; Dunker *et al.*, 1998, 2001, 2002a, b, 2005; Dunker and Obradovic, 2001; Dyson and Wright, 2005b; Tompa, 2002, 2005; Tompa and Csermely, 2004; Tompa *et al.*, 2005; Uversky, 2002a, b 2003b; Uversky *et al.*, 2000, 2005; Vucetic *et al.*, 2007; Wright and Dyson, 1999; Xie *et al.*, 2007a, b). Furthermore, sites of posttranslational modifications (acetylation, hydroxylation, ubiquitination, methylation, phosphorylation, etc.) and proteolytic attack are frequently associated with regions of intrinsic disorder (Xie *et al.*, 2007a). The

functional diversity provided by IDRs was suggested to complement functions of ordered protein regions (Vucetic *et al.*, 2007; Xie *et al.*, 2007a, b).

Another very important feature of the IDPs is their unique capability to fold under a variety of conditions (Dunker *et al.*, 2002a, 2005; Dunker and Obradovic, 2001; Dyson and Wright, 2002, 2005b; Fink, 2005; Iakoucheva *et al.*, 2002; Tompa, 2002; Uversky, 2002a, b; Uversky *et al.*, 2000, 2005; Wright and Dyson, 1999). In fact, the folding of these proteins can be brought about by interaction with other proteins, nucleic acids, membranes, or small molecules. It can also be driven by changes in the protein environment. The resulting conformations could be either relatively noncompact (i.e., remain substantially disordered) or tightly folded.

In a living organism, proteins participate in complex interactions, which represent the mechanistic foundation of the organism's physiology and function. Regulation, recognition, and cell signaling involve the coordinated actions of many players. To achieve this coordination, each participant must have a valid identification that is easily recognized by the other players. For proteins, these identification features are often located within IDRs (Dunker *et al.*, 2005; Uversky *et al.*, 2005). Despite (or may be due to) their high flexibility, IDPs are involved in regulation, signaling, and control pathways in which interactions with multiple partners and high-specificity/low-affinity interactions are often required (Dunker *et al.*, 2005; Uversky *et al.*, 2005).

IDPs have specific functions that can be grouped into four broad classes: (i) molecular recognition; (ii) molecular assembly; (iii) protein modification; and (iv) entropic chain activities (Dunker *et al.*, 2002a). Recently, the crucial role of intrinsic disorder in the action of RNA and protein chaperones was emphasized by showing that IDRs in these complex machines can function as molecular recognition elements that act as solubilizers by locally loosening the structure of the kinetically trapped folding intermediates (Tompa and Csermely, 2004).

1.1.2 Chaperones

1.1.2.1 Concept. Generally, a polypeptide chain of a protein contains all the information required to achieve the functional conformation (Anfinsen, 1973; Crick, 1958). Although this principle is generally correct for many foldable proteins, the information contained in some proteins is not sufficient to guarantee them the gain of functionally active structure. Such proteins cannot fold spontaneously and require the help of molecular chaperones. According to Ellis, molecular chaperones represent "a class of cellular proteins whose function is to ensure that the folding of certain other polypeptide chains and their assembly into oligomeric structures occur correctly" (Ellis, 1987). Chaperones are an important part of the cellular quality control system maintaining an intricate balance between protein synthesis and degradation and protecting cells from devastating consequences of uncontrolled protein aggregation. In addition to chaperones, this system includes the ubiquitin–proteasome system and the autophagy–lysosome system. Molecular chaperones protect cells from apoptosis induced by toxic

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oligomers. There are several mechanisms by which chaperones fight devastating consequences of misfolding and aggregation. These mechanisms can be grouped into three major classes of action: prevention, reversal, and elimination. At the prevention stage, chaperones bind to unfolded stretches in proteins and keep them in a folding-competent state while preventing aggregation. In the reversal mechanism, chaperones act as disaggregating and unfolding machines that help dissolve aggregates and give a misfolded protein a second chance for correct folding. At the elimination step, chaperones target misfolded proteins for degradation by the ubiquitin–proteasome system and/or the autophagy–lysosome system.

1.1.2.2 *Functional Classification of Chaperones.* The principal heatshock proteins (HSPs) that have chaperone activity belong to five conserved classes: HSP33, HSP60, HSP70, HSP90, HSP100, and the small heat shock proteins (sHsps). On the basis of their mechanism of action, molecular chaperones have been divided into three functional subclasses. "Folding" chaperones (e.g., DnaK and GroEL in prokaryotes, and Hsp60 and Hsp70 as well as the HspB group of Hsps including Hsp27 and HspB1 in eukaryotes) rely on adenosine triphosphate (ATP)-dependent conformational changes to mediate the net refolding/unfolding of their substrates. "Holding" chaperones (e.g., Hsp33 and Hsp31) bind partially folded proteins and maintain these substrates on their surface to await availability of "folding" chaperones. "Disaggregating" chaperones constitute the third class of chaperones (e.g., ClpB in prokaryotes and Hsp104 in eukaryotes), which promote the solubilization of proteins that have become aggregated as a result of stress.

According to their expression mechanisms, molecular chaperones are classified as inducible and constitutively expressed. Both types of chaperones act by selective binding of solvent-exposed hydrophobic segments of nonfolded polypeptides, and through multiple binding-release cycles bring about the folding, transport, and assembly of the target polypeptides (Bukau *et al.*, 2006; Hartl and Hayer-Hartl, 2002; Slepenkov and Witt, 2002b). Some chaperones are ATPases; that is, they use free energy from ATP binding and/or hydrolysis to perform work on their substrates.

The concentration of inducible chaperones, also known as HSPs, increases as a response to the stress conditions. Some of the illustrative examples of inducible chaperones are sHsps (e.g., α A-crystallin (HspB4), α B-crystallin (HspB5), Hsp27 (HspB1), and Hsp22 (HspB8); family of Hsp40; Hsp70 chaperones and their regulators-co-chaperones HDJ1, HDJ2, BAG1 (Bcl-2–associated athanogene), HSPBP1, Hip, Hop, and CHIP (carboxyl terminus of Hsc70-interacting protein); HspC group of Hsp including Hsp90, Grp94, Hsp104, and Hsp110. These molecular chaperones prevent and reverse the misfolding and aggregation of proteins, which occurs as a consequence of the stress (Lindquist, 1986; Lindquist and Craig, 1988).

On the other hand, constitutively expressed chaperones, also known as the heat shock cognate proteins (HSCs), facilitate protein translation, help newly synthesized proteins to fold, promote assembly of proteins into functional

complexes, and assist translocation of proteins into cellular compartments such as mitochondria and chloroplasts (Hartl and Hayer-Hartl, 2002; Young *et al.*, 2004). In the HSP70 family of proteins, in addition to the inducible Hsp70 form, there is a constitutively expressed form, the HSC (Hsc70), which has 85% identity with human Hsp70 and binds to nascent polypeptides to facilitate its correct folding. Hsc70 also acts as an ATPase participating in the disassembly of clathrin-coated vesicles during transport of membrane components through the cell (Goldfarb *et al.*, 2006).

Irrespective of being inducible or constitutively expressed, molecular chaperones evolve to protect proteins from misfolding and aggregation. An important feature of chaperones is that although they assist the noncovalent folding/unfolding and the assembly/disassembly of other macromolecular structures, they do not occur in these structures when the latter are performing their normal biological functions. Generally, molecular chaperones have no effect on protein folding rate. Of course, apparent folding and assembly rates can be increased by elimination of nonproductive oligomer/aggregate formation. Furthermore, by binding to partially folded species and preventing their aggregation, chaperones increase the yield of functional folded/assembled proteins. However, these actions do not affect the intramolecular folding rates. On the other hand, there is a last class of protein helpers that assist protein folding and are not present in the final folded/assembled functional form of a protein substrate. Therefore, these helpers known as *foldases* belong to the family of chaperones. Contrary to the typical chaperones considered so far, foldases evolve to catalyze the folding process by directly accelerating the protein folding rate-limiting steps. Among well-known foldases are eukaryotic protein disulfide isomerase (Goldberger et al., 1963; Hatahet et al., 2009; Nagradova, 2007), peptidyl-prolyl cis/transisomerase (Fischer et al., 1984; Nagradova, 2007), and lipase-specific foldases, Lifs, found in the periplasm of gram-negative bacteria (Jorgensen et al., 1991; Nagradova, 2007). Finally, there is a large class of the so-called intramolecular chaperones, which are specific protein regions, which are essential for protein folding but not required for protein function. Often, these N-terminal or C-terminal extensions are removed after the protein is folded by autoprocessing or by specific exogenous proteases (Chen and Inouye, 2008). On the basis of their roles in protein folding, intramolecular chaperones were classified into two categories. Type I category includes those intramolecular chaperones that assist tertiary structure formation and mostly are produced as the N-terminal sequence extension of the protein carrier. Type II category contains intramolecular chaperones that are not directly involved in tertiary structure formation but guide the assembly of quaternary structure to form the functional protein complex and are mostly located at the C-terminus of the protein carrier (Chen and Inouye, 2008).

1.1.3 Neurodegeneration

1.1.3.1 Concept. The term *neurodegeneration* is derived from the Greek word $v \varepsilon v \rho o$ -, *néuro*-, "nerval" and a Latin verb $d\overline{e}gener\overline{a}re$, "to decline" or

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"to worsen." Therefore, neurodegenerative diseases are a large class of human maladies, which includes various acquired neurological diseases with distinct phenotypic and pathologic symptoms, all characterized by the pathological conditions in which cells of the brain and/or spinal cord are lost. As the death of neurons increases, affected brain regions begin to shrink: by the final stage of Alzheimer's disease (AD), damage is widespread and the brain tissue has shrunk significantly; in prion disease, the brain undergoes damage known as *spongiform change* or *spongiosis* because when the tissue is examined under a microscope, it looks like a sponge, with many tiny holes.

Neurodegeneration is a slow process that begins long before the patient experiences any symptoms. It can take months or even years before visible outcomes of this degeneration are felt and diagnosed: in the case of AD, damage to the brain begins 10–20 years before any problems are evident. The progression through various AD stages may last from 8 to 10 years, whereas in Huntington disease (HD), death occurs approximately 18 years from the time of onset. Symptoms are usually noticed when many cells die or fail to function and a part of the brain begins to cease functioning properly. For example, the symptoms of Parkinson's disease (PD) become apparent after more than \sim 70% dopaminergic neurons die in a specific area of the midbrain known as *substantia nigra*.

As neurons are not readily regenerated, their deterioration over time leads to dysfunction and disabilities. Neurodegeneration, in principle, can affect various peripheral and central areas of the nervous system resulting in the great variability of the disease manifestations. Generally, neurodegenerative diseases can be divided into three groups according to their phenotypic effects: (i) conditions causing problems with movements; (ii) conditions affecting memory and leading to dementia; (iii) conditions affecting both movement and cognitive abilities; and (iv) conditions causing problems with peripheral nervous system.

Illustrative examples of movement neurodegenerative disorders include PD (characterized by symptoms originating from the neuronal loss in substantia nigra such as resting tremor on one (or both) side(s) of the body; generalized slowness of movement (bradykinesia); stiffness of limbs (rigidity); and gait or balance problems (postural dysfunction)); multiple system atrophy (MSA, characterized by several clinical features of PD); Kennedy disease (also known as spinal and bulbar muscular atrophy (SBMA) or X-linked spinal muscular atrophy since it affects the motor neurons of males only and characterized by muscle weakness); and various forms of ataxia (characterized by a failure of muscle coordination due to pathology arising in the spinocerebellar tract of the spinal cord).

Cognitive neurodegeneration is illustrated by AD and prion diseases (Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker (GSS) disease, fatal familial insomnia, and kuru). Some of the movement/cognition-affecting neurodegenerative diseases are neurodegeneration with brain iron accumulation type 1 (NBIA1, characterized by rigidity, dystonia, dyskinesia, and choreoathetosis (Malandrini *et al.*, 1996; Sugiyama *et al.*, 1993; Swaiman, 1991; Taylor *et al.*, 1996)), together with dysarthria, dysphagia, ataxia, and dementia (Dooling *et al.*, 1974; Jankovic *et al.*, 1985; Swaiman, 1991); dementia

with Lewy bodies (DLB, characterized by neuropsychiatric changes, often with marked fluctuations in cognition and attention, hallucinations, and parkinsonism (Galpern and Lang, 2006)); and HD (characterized by clinical effects on motor, cognitive, and psychological functions (Melone *et al.*, 2005)).

Illustrative examples of conditions with predominant involvement of the peripheral nervous system with minimal central nervous system involvement include pure autonomic failure (also known as *Bradbury–Eggleston syndrome* characterized by orthostatic hypotension leading to dizziness and fainting, visual disturbances, neck pain, chest pain, fatigue and sexual dysfunction (Hague *et al.*, 1997)), and Lewy body dysphagia (characterized by swallowing abnormalities caused by the localized Lewy body accumulation in both dorsal vagal motor nucleus and the nucleus ambiguus (Jackson *et al.*, 1995)).

1.1.3.2 Molecular Mechanisms of Neurodegeneration. Although neurodegenerative diseases are characterized by an extremely wide range of clinical symptoms resulting from dysfunction of different areas of the central and the peripheral nervous systems, the unifying mechanism of all these pathologies is the deterioration of specific regions of the nervous system caused by the highly specific and localized death of neurons. At the molecular level, many factors can induce neuronal death. Some of these factors are protein misfolding and aggregation, oxidative damage, mitochondrial dysfunction and impaired bioenergetics, disruption of neuronal Golgi apparatus and transport, and failure of cell protective mechanisms including chaperone system and impaired protein degradation machinery (e.g., proteasomal proteolysis and autophagy–lysosome system).

1.1.3.2.1 Protein Misfolding and Aggregation: Neurodegenerative Diseases as Proteinopathies and Amyloidoses. For a long time, a link between AD, PD, prion diseases, HD, and several other neurodegenerative disorders was elusive. However, recent advances in molecular biology, immunopathology, and genetics indicated that these diseases might share a common pathophysiologic mechanism, where derangement of a specific protein processing, functioning, and/or folding takes place. Therefore, neurodegenerative disorders represent a set of proteinopathies, which can be classified and grouped on the basis of the causative proteins. In fact, from this viewpoint, neurodegenerative disorders represent a subset of a broader class of human diseases known as *protein confor*mational or protein misfolding diseases. These disorders arise from the failure of a specific peptide or protein to adopt its native functional conformational state. The obvious consequences of misfolding are protein aggregation (and/or fibril formation), loss of function, and gain of toxic function. Some proteins have an intrinsic propensity to assume a pathologic conformation, which becomes evident with aging or at persistently high concentrations. Interactions (or impaired interactions) with some endogenous factors (e.g., chaperones, intracellular or extracellular matrixes, other proteins, and small molecules) can change conformation of a pathogenic protein and increase its propensity to misfold. Misfolding can originate from point mutation(s) or result from an exposure to internal

or external toxins, impaired posttranslational modifications (phosphorylation, advanced glycation, deamidation, racemization, etc.), an increased probability of degradation, impaired trafficking, lost binding partners, or oxidative damage. All these factors can act independently or in association with one another.

Many of the neurodegenerative diseases are in fact protein deposition diseases. In other words, they are associated with the formation of extracellular amyloid fibrils or intracellular inclusions with amyloid-like characteristics. Protein deposition diseases can be sporadic (idiopathic, 85%), hereditary (familial or genetically inherited, 10%), or even transmissible, as in the case of prion diseases (5%) (Chiti and Dobson, 2006). In the first case, neurodegeneration develops spontaneously, without obvious alterations in the patient's DNA (although genetic differences may act as risk factors). In the second case, neurodegeneration is caused by mutation(s) in specific gene(s). Although these diseases are very different clinically, they share similar molecular mechanisms where a specific protein or protein fragment changes from its natural soluble form into insoluble fibrils. It has been pointed out that prior to fibrillation, amyloidogenic polypeptides may be rich in β -sheet, α -helix, β -helix, or contain both α -helices and β -sheets. They may be globular proteins with rigid 3D structure or belong to the class of natively unfolded (or intrinsically unstructured) proteins (Uversky and Fink, 2004). Despite these differences, the fibrils from different pathologies display many common properties, including a core cross- β -sheet structure in which continuous β -sheets are formed, with β -strands running perpendicular to the long axis of the fibrils (Sunde *et al.*, 1997). This β -pleated sheet structure of fibrils constitutes the basis of the unusual resistance of all kinds of amyloid to degradation and, therefore, the progressive deposition of the material (Westermark, 2005). Furthermore, all fibrils have similar twisted, rope-like structures that are typically 7-13 nm wide (Serpell et al., 2000; Sunde and Blake, 1997) and consist of a number of protofilaments (typically 2-6), each about 2-5 nm in diameter (Serpell *et al.*, 2000). Alternatively, protofilaments may associate laterally to form long ribbons that are 2-5 nm thick and up to 30 nm wide (Bauer *et al.*, 1995; Pedersen et al., 2006; Saiki et al., 2005).

Although amyloid-like fibrils are frequently observed in several neurodegenerative diseases and although the importance of specific amyloidogenic proteins in etiology of corresponding diseases was established by multiple genetic and pathological studies, there is no unifying model explaining toxicity of these deposits. In fact, several different mechanisms of toxicity have been proposed on the basis of the monomeric/polymeric nature of the proposed toxic species. Let us consider the role of α -synuclein in the pathology of PD as an illustrative example, for which at least three different mechanisms of neurotoxicity were discussed (Waxman and Giasson, 2009). An increase in intracellular abundance of monomeric α -synuclein has been considered as a potential cause of neuronal toxicity. This hypothesis is supported by the fact that 50% or 100% increase in α -synuclein expression caused by the duplication or triplication of the α -synuclein gene is known to result in familial forms of PD or DLB (Ross *et al.*, 2008). Furthermore, increased α -synuclein expression was reported in specific brain areas or

types of neurons in individuals with sporadic PD (Dachsel et al., 2007) as well as in brains of model animals as a result of toxic insult (Goers et al., 2003; Manning-Bog et al., 2002). In another model, specific oligometric and protofibrillar forms of a-synuclein have been proposed as potent toxic species. Here, α -synuclein oligometric were proposed to form pores on intracellular membranes such as the plasma membrane and may increase cation permeability (Ding et al., 2002; Lashuel et al., 2002; Volles et al., 2001). Finally, it was emphasized that the fibrillation of α -synuclein and formation of large intracytoplasmic inclusions that can cause the dysfunction and the demise of neurons or oligodendrocytes (Waxman and Giasson, 2009). These inclusions may act as "sinks," recruiting other necessary, cellular proteins from their normal cellular functions (Waxman and Giasson, 2009). They may affect proteasome function (Lindersson et al., 2004) and can impair cellular functions by obstructing normal cellular trafficking (including disruption of endoplasmic reticulum (ER) and Golgi apparatus), by disrupting cell morphology, by impairing axonal transport, and by trapping cellular components (e.g., mitochondria) (Waxman and Giasson, 2009). Of course, the discussed mechanisms of α-synuclein toxicity based on the different polymeric forms from small oligomers to amyloid fibrils are not necessarily mutually exclusive because the presence of any polymeric form of α -synuclein is abnormal and may be problematic for the normal activities of cells, thereby resulting in neurodegeneration (Waxman and Giasson, 2009).

1.1.3.2.2 Mitochondrial Dysfunction and Impaired Bioenergetics. Mitochondria, in addition to being a source of ATP, perform pivotal biochemical functions necessary for homeostasis and represent a convergence point for both extracellular and intracellular death signals. Mitochondrial dysfunction has been described in several neurodegenerative diseases including AD, PD, HD, and amyotrophic lateral sclerosis (ALS) (Moreira et al., 2010). For example, in AD brains, the impaired activity of three tricarboxylic acid cycle complexes, pyruvate dehydrogenase, isocitrate dehydrogenase, and α -ketoglutarate dehydrogenase, was observed (Bubber et al., 2005) together with the reduced respiratory chain activities in complexes I, III, and IV (Valla et al., 2006) and the presence of alterations in mitochondria morphology and distribution (Wang et al., 2008). In PD, mitochondria were demonstrated to be one of the direct targets of a-synucleintriggered toxicity, which that caused reduced mitochondrial complex I activity and increased production of reactive oxygen species (ROS; Devi et al., 2008). Furthermore, in both sporadic and familial forms of PD, reported mitochondrial abnormalities include impaired functioning of the mitochondrial electron transport chain, aging-associated damage to mitochondrial DNA, impaired calcium buffering, and anomalies in mitochondrial morphology and dynamics (Banerjee et al., 2009; Gibson et al., 2010). Reductions in the activities of complexes II, III, and IV have been observed in the caudate and putamen of HD patients (Browne et al., 1997). Finally, in ALS, the presence of mutant Cu/Zn superoxide dismutase (SOD1) within motor neurons was shown to cause alterations of

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the mitochondrial respiratory chain (Dupuis *et al.*, 2004), specifically affecting performance of the mitochondrial complexes II and IV (Zimmerman *et al.*, 2007).

1.1.3.2.3 Oxidative Damage. There are several factors that put the brain at risk from oxidative damage (Fatokun *et al.*, 2008). Some of these factors include high oxygen consumption (20% of the total basal O_2 consumption of the body), critically high levels of both iron and ascorbate, relatively low levels of antioxidants (e.g., catalase), a tendency to accumulate metals with age, and low regenerative capacity (Barnham *et al.*, 2004; Gaeta and Hider, 2005; Halliwell, 2006). Furthermore, microglia, the resident immune cells of the brain, produce superoxide and H₂O₂ upon activation; they also produce cytokines that can enhance production of ROS and NO (Halliwell, 2006). Astrocytes equally produce cytokines through which they can be activated to generate NO from iNOS (Halliwell, 2006). The microglia and astrocytes are therefore major mediators of inflammatory processes in the brain (Duncan and Heales, 2005). Some cytochromes *P*450 are also a source of ROS in certain brain regions (Gonzalez, 2005).

Therefore, it is not surprising that although the etiology, symptoms, and disease localization are not the same for neurodegenerative diseases, oxidative stress is recognized as an important pathway leading to neuronal death and is implicated in many neurodegenerative diseases including AD, PD, HD, ALS, and Friedreich's ataxia (FA) (Barnham et al., 2004; Fatokun et al., 2008; Qureshi and Parvez, 2007). In AD, the major sources of oxidative stress and free radical production are copper and iron when bound to $A\beta$, and the various forms of $A\beta$ in the AD brain are commonly found to be oxidatively modified (Barnham *et al.*, 2004). In PD, resulting from selective degeneration of neuromelanin-containing neurons, most notably the nigral dopaminergic neurons, the catechol dopamine can generate H₂O₂ and the oxidative stress could come from a failure to regulate dopamine-iron biochemistry (Barnham et al., 2004). In ALS, the mutations in SOD are known to lead to a toxic gain of function promoting a pro-oxidant activity of SOD generating ROS (Barnham et al., 2004). FA originates because of an abnormal GAA trinucleotide expansion within the gene encoding the mitochondrial protein frataxin, causing frataxin deficiency. Iron therefore accumulates in the mitochondria, promoting oxidative stress that leads to cardiomyopathy and neurodegeneration (Barnham et al., 2004).

1.1.3.2.4 Disruption of Neuronal Golgi Apparatus and Impaired Transport.

The Golgi apparatus plays a central role in the transport, processing, and sorting of proteins. The complex consists of stacks of parallel cisternae and vesicles that carry molecular "cargo" from one cisterna to the next by the coordinated fission of vesicles from the lateral edge of one cisterna and fusion to the next cisterna (Rambourg and Clermont, 1997). Interactions between amyloidogenic proteins and any one or more proteins involved in the maintenance of the structure of the Golgi apparatus might disrupt its structure and function. Golgi apparatus fragmentation was reported in ALS, corticobasal degeneration, AD, PD, CJD, and

in spinocerebellar ataxia type 2 (SCA2). In mice model of familial ALS, fragmentation of the Golgi apparatus of spinal cord motor neurons and aggregation of mutant protein were detected months before the onset of paralysis (Gonatas *et al.*, 2006). In a cellular PD model, cells with prefibrillar α -synuclein aggregates had fragmented Golgi apparatus and showed trafficking impairment. These results strongly suggested that the fragmentation of the Golgi apparatus is an early event that occurs before the appearance of the fibrillar α -synuclein forms (Gosavi *et al.*, 2002).

1.1.3.2.5 Impaired Protein Degradation Machinery. The proteasome, in collaboration with a sophisticated ubiquitin system used for marking target proteins, selectively degrades short-lived regulatory proteins as well as abnormal proteins that must be eliminated from cells. The lysosome-linked autophagy system is a bulk protein degradation system designed to eliminate cytoplasmic constituents and to play a prominent role in starvation response and quality control of organelles in cells. The majority of characteristic proteinaceous inclusions in AD, PD, ALS, and frontotemporal lobar degeneration (FTLD) are ubiquitinpositive (Alves-Rodrigues *et al.*, 1998; Lim, 2007). This clearly suggests that impaired proteasomal proteolysis is the main mechanism for the accumulation of ubiquitinated proteins and inclusion body formation in many neurodegenerative diseases (Matsuda and Tanaka, 2010).

Furthermore, since ubiquitination is recently recognized as a mechanism relevant to the autophagy–lysosome system, the fact that specific inclusions in neurodegenerative diseases are ubiquitinated may reflect the impairment of this degradation system too. In fact, the autophagosome sequesters cytosolic material nonspecifically and therefore for a long time the autophagic degradation was considered as a nonselective process. However, recent studies clearly showed that several subcellular structures such as mitochondria and protein aggregates are degraded by selective autophagy and that ubiquitin is involved in this process (Ishihara and Mizushima, 2009; Kirkin *et al.*, 2009). Later, the impairment of the autophagy system in neurons was shown to cause neurodegeneration and ubiquitin-positive inclusion formation in mice (Hara *et al.*, 2006; Komatsu *et al.*, 2006).

1.1.3.2.6 Chaperone System Dysfunctions. Maintaining the appropriate intracellular complement of functional proteins depends on the robust, well-organized, and self-regulated protein quality control system that maintains a balance between protein synthesis and degradation and is capable of a targeted response if an imbalance occurs where misfolded, aggregated, or otherwise damaged proteins accumulate (Bukau *et al.*, 2006; Leidhold and Voos, 2007; McClellan *et al.*, 2005; Witt, 2010). This system tags misfolded and aggregated proteins for refolding by molecular chaperones or degradation by protein degradation machinery such as the ubiquitin-dependent proteasome system or the lysosome-linked autophagy system (Goldberg, 2003). The first line of defense against protein misfolding and aggregation are molecular chaperones. Although,

under normal conditions, any protein can spontaneously misfold and aggregate, the "nonstress" concentration of such misfolded, aggregated, or amyloid proteins is negligible and these potentially toxic species are efficiently eliminated by the quality control system. However, several conditions are known to promote protein misfolding and aggregation. This includes the classic environmental stresses such as heat and cold, heavy metals, toxic chemical compounds, UV radiation, the synthesis of proteins with mutations, and age-related decrements in the protein quality control system itself. The enhanced misfolding and aggregation result in the abuse and potential failure of the quality control system. In its turn, the failure of this protein quality control system to fulfill its functions or malfunction of either one or both of its components generates the potential for tissue-specific buildup of protein aggregates termed *amyloid* and is related to the development of neurodegenerative or "conformational" diseases (Gao and Hu, 2008). More details of chaperone action in neurodegeneration together with the description of the role of intrinsic disorder in their activities are given in the next section of this chapter.

1.2 INTRINSICALLY DISORDERED CHAPERONES IN NEURODEGENERATION

As mentioned above, molecular chaperones play a number of important roles in fighting protein misfolding and aggregation and therefore in protecting neurons from the cytotoxic effects of misfolded/aggregated species. This neuroprotection involves a highly coordinated and orchestrated action of multiple players. Therefore, there is an entire net of macromolecular chaperones and their helpers, co-chaperones. The detailed description of individual chaperones and their role in neuroprotection are covered in subsequent chapters of this book. Earlier, it has been emphasized that the importance of intrinsic disorder for the function of chaperones can be underlined by the analysis of the abundance of predicted intrinsically disordered residues in chaperones (Tompa and Csermely, 2004). This analysis revealed a high proportion of such regions in protein chaperones, 36.7% residues of which fall into disordered regions and 15% fall within disordered regions longer than 30 consecutive residues (Tompa and Csermely, 2004). The major goal of this section is to show that many neuroprotective chaperones/cochaperones are either completely disordered or possess long disordered regions and to emphasize that intrinsic disorder plays a crucial role in their action. Corresponding information is provided for the Hsp70 system, the Hsp90 system, several sHsps, and members of the synuclein family.

1.2.1 The Hsp70 System

1.2.1.1 Major Players. Hsp70 is a 70-kDa molecular machine that is able to interact with exposed hydrophobic amino acids in various polypeptides, hydrolyzes ATP, directs its substrates into a variety of distinct fates, and therefore acts at multiple steps in a protein's life cycle, including its folding, trafficking,

remodeling, and degradation (Bukau et al., 2006; Frydman, 2001; Genevaux et al., 2007; Mayer and Bukau, 2005; Patury et al., 2009). Since Hsp70 is able to bind promiscuously, it is considered now as a core chaperone for the proteome (Erbse et al., 2004; Rudiger et al., 1997a, b) and a central mediator of protein homeostasis. The activity of Hsp70 is known to be modulated by a number of co-chaperones, which bind to the core chaperone and influence its functions. Among the most important Hsp70 co-chaperones are various J-domain proteins (e.g., HDJ1 and HDJ2), the number of nucleotide exchange factors (NEFs such as GrpE, Bag1, Hsp110, and HspBP1), and several tetratricopeptide repeat (TPR) co-chaperones (e.g., Hip). Mammalian cells contain a large net of various Hsp70s and their decorating proteins: there are approximately 13 Hsp70s, >40 J-domain proteins, at least 4 distinct types of NEFs, and dozens of proteins with TPR domains. Since at any given time, an individual Hsp70 molecule can only interact with a single representative of each major co-chaperone class, this means that tens of thousands of possible chaperone-co-chaperone complexes might be formed in the cell (Patury et al., 2009).

Finally, there are also several co-chaperones connecting Hsp70 to the Hsp90 and proteasomal degradation pathways. For example, the Hsp-organizing protein (Hop) mediates interactions between Hsp70 and Hsp90 (Scheufler *et al.*, 2000). Hsp70 and Hsp90 also bind to a protein co-chaperone CHIP (Ballinger *et al.*, 1999; Connell *et al.*, 2001), which is a member of the family of E3 ubiquitin ligases. CHIP ubiquitinates unfolded proteins bound to Hsp70 and Hsp90, and these tagged proteins are degraded by the proteasome. Therefore, CHIP links Hsp70 and Hsp90 chaperones to the proteasomal degradation pathway (Witt, 2010).

1.2.1.2 Hsp70. Hsp70 is a highly abundant ($\sim 1-2\%$ of total cellular protein) and highly conserved protein, with \sim 50% sequence identity between prokaryotic and mammalian family members. Many organisms express multiple Hsp70s (e.g., 13 in humans), and members of this class of chaperones are found in all the major subcellular compartments (Patury et al., 2009). Hsp70 is composed of three major domains: an \sim 44-kDa N-terminal nucleotide-binding domain (NBD, residues 1-388), an ~15-kDa substrate-binding domain (SBD, residues 393-537), and an ~10-kDa C-terminal α -helical, "lid" domain (residues 538–638). All three domains are important for the function of Hsp70. NBD competitively binds ATP and adenosine diphosphate (ADP) and can slowly hydrolyze ATP (McCarty et al., 1995). SBD binds target peptide via the hydrophobic substrate-binding cleft. NBD and SBD are connected by a hydrophobic linker that is crucial for the functional association of two domains: when ATP is bound to NBD, the SBD and NBD exhibit coupled motion, suggesting their tight association (Bertelsen et al., 2009; Schuermann et al., 2008). The position of the lid domain regulates the accessibility of the peptide-binding site. In the ATP-bound form, the lid domain remains open, which facilitates transient interactions with substrates. Following ATP hydrolysis, a conformational change releases the SBD, resulting in closure of the lid and an \sim 10-fold increase in the affinity for substrate (Slepenkov and Witt, 2002a; Wittung-Stafshede et al., 2003). An important feature of the ATP

binding to Hsp70 is that this chaperone binds ATP tightly ($K_d = 1$ nM) but hydrolyses it very slowly ($k_{hy}^{app} = 3 \times 10^{-4} \text{ s}^{-1}$ at 25°C) (Russell *et al.*, 1998). Another important feature of these chaperones is that the nucleotide modulates their peptide binding and release; in the absence of co-chaperones, ADP-bound DnaK binds and releases peptides over a timescale of minutes or even hours, whereas ATP-bound DnaK binds and releases peptides over a timescale of seconds or even milliseconds (Slepenkov and Witt, 2002a; Wittung-Stafshede *et al.*, 2003). Overall, HSP70s function in a dynamic cycle of binding and releasing polypeptide substrate coupled to a cycle of ATP binding and hydrolysis by the intramolecular ATPase. In the ATP-bound state, HSP70s exhibit fast kinetics and low affinity for polypeptide substrates, whereas in the ADP-bound state, this chaperone exhibits slow kinetics and high affinity for polypeptide substrates. These cycling states are highly regulated by at least five different co-chaperones: Hsp40, Hip, Hop, Bag-1 (Bcl2-associated athanogene 1 or RAP46), and CHIP (Shi *et al.*, 2007).

The substrates of Hsp70 proteins are unrelated in sequence and structure and represent a large spectrum of folding conformers of foldable proteins. In addition, Hsp70 interacts indiscriminately with nonnative polypeptides such as a broad spectrum of heat-denatured proteins (Wegele *et al.*, 2004). The broad substrate specificity of Hsp70s implies a rather degenerative binding motif. It was shown by NMR and X-ray crystallography that DnaK binds its substrate in an extended conformation (Wegele *et al.*, 2004).

The data above clearly show that action of HSP70s involves a lot of dynamics. To test how this functional dynamics correlates with the intrinsic disorder status, DnaK sequence was analyzed by several disorder predictors. DnaK was chosen as the illustrative member of Hsp70 family. Figure 1.1 represents the distribution of the intrinsic disorder propensity within the DnaK sequence evaluated by PONDR VSL2 and illustrates that all three domains of the protein contain extensive amounts of intrinsic disorder. The mean disorder propensity within these domains is arranged in the following order: NBD $(0.36 \pm 0.13) < \text{SBD}$ $(0.49 \pm 0.19) < \text{lid} (0.84 \pm 0.08)$, with the C-terminal lid domain predicted to be entirely disordered. This high intrinsic propensity of the protein for intrinsic disorder is crucial for its function. It is also reflected in the fact that for a very long time, the 3D structure of a full-length Hsp70 chaperone has not been resolved and all structural information about this important protein was derived from the analysis of its separate domains obtained from partial proteolysis (Slepenkov and Witt, 2002a, 2002b). Furthermore, even when structures comprising both the NBD and the SBD have become available, none of these structures was compatible with any of the others. The location where the SBD docks to the NBD differs by tens of Angstroms (Bertelsen et al., 2009).

Recently, the solution conformations for the full-length, *Escherichia coli* DnaK (1–638) and for a truncation (1–605) for the chaperone bound to substrate peptide (NRLLLTG) and ADP were determined by NMR techniques (Bertelsen *et al.*, 2009). The analysis revealed that although the NBD, SBD, and linker move relatively independently of each other, the motion of SBD with respect





Figure 1.1 The distribution of the intrinsic disorder propensity within the DnaK sequence evaluated by PONDR VSL2. Positions of the three domains, NBD (residues 1–388), SBD (residues 389–537), and lid (538–638) are also shown. All domains contain a significant amount of disorder, with the C-terminal lid domain predicted to be entirely disordered.

to NBD is restricted to a cone of $\sim 70^{\circ}$ opening angle. However, within this cone, there was a preferred orientation of SBD with respect to NBD that can be defined with a relatively high precision (Bertelsen *et al.*, 2009). Importantly, NBD–SBD linker residues (379–397) were shown to possess a large amount of flexibility for these residues, and the lack of dispersion in NMR chemical shifts suggested that this flexible linker had a random coil conformation (Bertelsen *et al.*, 2009). On the other hand, SBD and lid domains were shown to move together as a single rigid unit in the ADP–peptide state, and residues 606–638 were disordered in solution (Bertelsen *et al.*, 2009).

1.2.1.3 Hsp70 Co-chaperones

1.2.1.3.1 J-Domain Proteins. The members of the J protein family, also known as the Hsp40 family (or DnaJ-related co-chaperones), are highly diverse and range in size from 116 amino acids (DnaJC19) to 2243 amino acids (DnaJC13 or Rme-8) (Gibbs and Braun, 2008). In humans, there are over 44 J proteins with the only common feature of this family being a conserved \sim 70 amino acid signature region known as a *J domain* (Zhao *et al.*, 2008). Although J proteins are known to modulate the Hsp70 ATP catalytic activity via their conserved J domain, all these proteins have multiple additional domains with various functionalities, clearly reflecting the functional diversity of this family probably related to the client protein recognition (Gibbs and Braun, 2008). Therefore, J proteins serve as regulators of the ATPase activity and substrate-binding specificity of Hsp70s. Hsp40 proteins are classified into

three main subfamilies (A-C), also referred to as types I-III) (Cheetham and Caplan, 1998; Mayer et al., 2001; Ohtsuka and Hata, 2000). Subfamily A consists of proteins with the four domains: the highly conserved α -helical N-terminal domain, referred to as the J domain (Greene et al., 1998; Karzai and McMacken, 1996; Laufen et al., 1999; Suh et al., 1999; Szabo et al., 1994), a glycine/phenylalanine-rich region that is disordered and likely to be responsible for flexibility (Karzai and McMacken, 1996; Szyperski et al., 1994), the central cysteine-rich domain that includes four repeats of the motif CXXCXGXG (where X is any amino acid) and folds in a zinc-dependent manner with two repeats bound to one zinc ion (Banecki et al., 1996; Martinez-Yamout et al., 2000; Szabo et al., 1996), and the C-terminal domain that forms a β -sheet structure and is involved in the dimerization of Hsp40 (Sha et al., 2000). The Cys-rich and C-terminal domains are involved in substrate binding and presentation (Banecki et al., 1996; Li et al., 2003; Lu and Cyr, 1998). Subfamily B contains proteins that lack the Cys-rich domain, and subfamily C has only the J domain, which is not necessarily located at the N terminus (Cheetham and Caplan, 1998; Mayer et al., 2001; Ohtsuka and Hata, 2000).

Results of the disorder prediction for illustrative members of J proteins from classes A (human DnaJ homolog subfamily A member 1), B (human DnaJ homolog subfamily B member 1), and C (human DnaJ homolog subfamily C member 21) are shown in Figure 1.2. All three co-chaperones are predicted to be highly disordered, possessing averaged disorder scores of 0.60 ± 0.20 , 0.54 ± 0.23 , and 0.74 ± 0.28 , respectively. The fact that all three proteins contain long disordered regions is further confirmed by the lack of the resolved 3D structures of the full-length proteins.

1.2.1.3.2 TPR Co-Chaperone Hip. Hip (Hsp70-interacting protein) is a 369amino acid cytosolic protein that is composed of an N-terminal region (residues 1-100, which is responsible for protein homo-oligomerization (Velten *et al.*, 2000)), a central, TPR domain (residues 114–215), followed by a highly charged region (residues 230–272), and a C-terminal region (residues 283–369) containing GGMP repeats and a Stil domain (heat shock chaperonin-binding motif) (Shi et al., 2007). Hip plays a crucial role in the Hsp70 cycle. In fact, the initial Hsp70 interaction with a polypeptide substrate is achieved through chaperone cooperation with a member of the Hsp40 family, the binding of which stimulates the ATPase activity of Hsp70 and generates the high affinity, ADP-bound state. However, in the presence of Hsp40 alone, the ADP state of Hsp70 is unstable, and the newly formed Hsp70-substrate complex may dissociate prematurely (Hohfeld et al., 1995). The TPR domain of Hip binds to the HSP70 ATPase domain and stabilizes the ADP-bound state of Hsp70, thus stabilizing the chaperone-substrate complex. TPR domain and flanking highly charged region are required for Hip to bind the HSP70 ATPase domain (Hohfeld et al., 1995). Figure 1.3a presents disorder prediction for human Hip co-chaperone and shows that this protein is predicted to be intensively disordered.



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Figure 1.2 Intrinsic disorder in illustrative members of the J proteins (Hsp40s) cochaperones from the classes A ((a) human DnaJ homolog subfamily A member 1), B ((b) human DnaJ homolog subfamily B member 1), and C ((c) human DnaJ homolog subfamily C member 21). The localizations of major domains in these Hsp40 proteins are also indicated.

1.2.1.3.3 NEF Co-Chaperone Hsp110. Hsp110 proteins constitute a heterogeneous family of abundant molecular chaperones, which are found exclusively in the cytosol of eukaryotic organisms and are evolutionarily related to the Hsp70 family (Easton *et al.*, 2000). Hsp100 was shown to reside in a large molecular complex that includes Hsp70 and Hsp25 (Wang *et al.*, 2000). The N-terminal



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Figure 1.3 Predicted intrinsic disorder in the co-chaperones of the Hsp70 machinery: (a) Disorder prediction for human Hip co-chaperone; (b) disorder in the NEF co-chaperone, human Hsp70-related protein APG-2 (human Hsp110); (c) intrinsic disorder prediction in the human BAG family molecular chaperone regulator 1L (BAG-1L); and (d) predicted disorder in the human CHIP also known as the STIP1 homology and U box-containing protein 1. The localizations of major functional domains in these proteins (when known) are also indicated.

ATPase domain of Hsp110 proteins possesses significant amino acid sequence homology with the Hsp70 proteins, whereas sequence homology between these proteins in their C-terminal domains is very low and hardly recognizable. The C-terminal domain of Hsp100s is considerably longer than that of classical Hsp70 proteins, partially because of a highly negatively charged insertion characteristic for Hsp110 proteins (Oh *et al.*, 1999). Members of the Hsp110 family are known to be efficient "holdases"—they prevent the aggregation and assist the refolding of heat-denatured model substrates in the presence of Hsp70 chaperones and their co-chaperones (Raviol *et al.*, 2006a). It has been shown recently that the Hsp110 from the yeast, Sse1p, acts as an efficient NEF for the yeast cytosolic Hsp70s, Ssa1p, and Ssb1p (Raviol *et al.*, 2006b). Figure 1.3b shows that human Hsp70-related protein APG-2 (human Hsp110) possesses a significant amount of intrinsic disorder, especially in its C-terminal domain. In fact, the mean disorder score for this protein is 0.52 ± 0.28 , whereas its last 380 residues are characterized by the disorder score of 0.76 ± 0.19 .

1.2.1.3.4 NEF Co-chaperone BAG1. BAG-1 is a multifunctional protein implicated in the modulation of a large variety of cellular processes ranging from transcriptional regulation, to the regulation of apoptosis, to the control of cell migration (Alberti et al., 2003; Doong et al., 2002; Takayama and Reed, 2001). In relation to the chaperone system, BAG-1 is intimately involved in the regulation of Hsp70 chaperone proteins in the eukaryotic cytosol and nucleus, thereby modulating the Hsp70-mediated protein folding and degradation pathways (Alberti et al., 2003). In fact, BAG-1 is known to stimulate nucleotide exchange on mammalian cytosolic Hsc70 (Hohfeld and Jentsch, 1997). There are at least four isoforms of BAG-1: BAG-1L (apparent molecular mass of 52 kDa), BAG-1M (46 kDa; also termed HAP46, RAP46), BAG-1 (34 kDa), and BAG-1S (29 kDa) (Takayama et al., 1998). The major difference between these isoforms is in their N-terminal domains, which differ in their content of several structural elements, the presence or absence of which brings functional diversity to distinct isoforms (Alberti et al., 2003). In addition to the conserved BAG domain located at the C-terminus of BAG-1s, all isoforms contain the ubiquitin-like domain, which serves as an integral sorting signal to stimulate an interaction of BAG-1 with the proteasome and therefore provide a unique link between the Hsp70 and ubiquitin-proteasome systems (Alberti et al., 2003). Furthermore, human cells contain several BAG-1-related proteins: BAG-2, BAG-3 (CAIR-1; Bis), BAG-4 (SODD), BAG-5, and BAG-6 (Scythe, BAT3), which in addition to the conserved BAG domain required for binding and regulation of Hsc70, possess various functional domains that mediate their targeting to diverse partner proteins and subcellular compartments (Takayama and Reed, 2001). The plethora of biological functions ascribed to BAG-1 can be understood by taking into account the fact that this protein is highly disordered. Intrinsic disorder distribution within the sequence of the human BAG family molecular chaperone regulator 1L (BAG-1L) is shown in Figure 1.3c.

1.2.1.3.5 Carboxyl Terminus of Hsc70 Interacting Protein (CHIP). Similar to BAG-1, CHIP (carboxyl terminus of Hsc70 interacting protein) contains both a chaperone-binding site and a domain implicated in the regulation of the ubiquitin-proteasome system. CHIP interacts with binding sites for TPRcontaining co-chaperones Hsc70 and Hsp90 via a tandem of three TPR motifs at its amino terminus, whereas at its carboxyl terminus, this protein contains a U-box, which is structurally related to RING finger domains found in many ubiquitin ligases (Jackson et al., 2000). CHIP by itself possesses ubiquitin ligase activity and, in coordination with ubiquitin-conjugating enzymes of the Ubc4/5 family, mediates ubiquitin attachment to protein substrates bound by Hsc70 and Hsp90 (Ballinger et al., 1999; Murata et al., 2001). Since Hsc70 contains the nonoverlapping binding sites for BAG-1 and CHIP, these two factors can simultaneously associate with the chaperone (Ballinger et al., 1999). Furthermore, in the ternary BAG-1/Hsc70/CHIP complex, CHIP mediates the attachment of a polyubiquitin chain to BAG-1 promoting the association of the chaperone complex with the proteasome, thereby providing the mechanism of the chaperone-assisted degradation pathway regulation (Alberti et al., 2002). In application to neurodegeneration, it has been recently established that CHIP targets the toxic α -synuclein oligomers for degradation (Tetzlaff et al., 2008). Figure 1.3d represents the results of disorder prediction for human CHIP, also known as the STIP1 homology, and U-box-containing protein 1. Figure 1.3d shows that although TPR-containing domain and U-box domain are predicted to be mostly ordered, they are connected by a long, highly disordered linker. The flexibility of this linker very likely helps in decoupling CHIP interactions with BAG-1 and Hsp70.

1.2.1.3.6 Co-Chaperone Hop. Hop (Hsp70/Hsp90-organization protein, also known as stress-induced phosphoprotein 1, STIP1) does not act as chaperone by itself (Bose et al., 1996). However, it is involved in the organization of Hsp70/Hsp90 complex via its three TPR domains serving as nonoverlapping binding sites for both Hsp70 and Hsp90. In fact, the EEVD-containing C-termini of Hsp70 and Hsp90 bind specifically to the Hop TPR domains, TPR1, and TPR2a, respectively (Scheufler et al., 2000). The connection of and the interplay between the Hsp70 and Hsp90 chaperone machineries is of crucial importance for cell viability. Although originally Hop was considered as a linker protein that brings and holds together Hsp70 and Hsp90 (Smith et al., 1993), the functional repertoire of this co-chaperone is essentially broader, since Hop is involved in regulation of activities of these two chaperones (Odunuga et al., 2004). The chaperone activities of both Hsp70 and Hsp90 are dependent on their ability to bind and hydrolyze ATP. These two chaperones are constantly recycled between the ADP- and ATP-bound forms. The ATPase activity of either Hsp70 or Hsp90 can be divided mechanistically into two stages: ATP hydrolysis and ADP/ATP (nucleotide) exchange. Hop serves as one such modulator. Hsp40 enhances the binding of Hsp70 to preexisting Hop-Hsp90 complex by stimulating the conversion of Hsp70-ATP to Hsp70-ADP (Hernandez et al., 2002). Human Hop

binds to Hsp70 with low affinity, but the strength of interaction increases in the presence of Hsp90 (Hernandez *et al.*, 2002). Despite noticeable sequence homology between human Hop and its yeast homolog Sti1 (37% identity), there are fundamental differences between these two proteins in the regulation of the mammalian Hsp90 system compared to the yeast complex (Wegele *et al.*, 2003). In the mammalian system Hop has no influence on the ATPase activity of the Hsp70 or Hsp90 component (Wegele *et al.*, 2003), whereas Sti1 is a noncompetitive inhibitor of yHsp90 (Richter *et al.*, 2003) and a potent activator of yHsp70 (Wegele *et al.*, 2003).

Hop exists as a dimeric molecule in solution and binds as a dimer to dimeric Hsp90 (Carrigan *et al.*, 2004). Structurally, Hop is defined by the presence of nine TPR motifs (which are loosely conserved repeats of roughly 34 amino acids known to mediate protein–protein interactions) clustered into three TPR domains, each consisting of three TPR motifs. A TPR motif shows a helix-turnhelix structure and subsequent TPR motifs are ordered in antiparallel α -helices (Das *et al.*, 1998). Each TPR domain is able to form a structural module that directs protein–protein interactions and has been recruited by different proteins and adapted for various protein–protein interaction functions (Blatch and Lassle, 1999). Owing to its TPR domains, Hop participates in the formation of several Hsp70/Hsp90-unrelated complexes, for example, serving as a receptor for prion proteins (Odunuga *et al.*, 2004). The results of disorder prediction in human Hop (STIP1) are shown in Figure 1.4.



Figure 1.4 The distribution of the intrinsic disorder propensity within the sequence of the human co-chaperone Hop (Hsp70/Hsp90-organization protein, also known as stress-induced-phosphoprotein 1, STIP1) evaluated by PONDR VSL2. The localizations of major domains in this co-chaperone are also indicated.

1.2.2 The Hsp90 Chaperone System

1.2.2.1 Major Players. Although the Hsp70 machinery is one of the most frequently used folding systems in the cell, which is responsible for the correct folding of a wide variety of protein substrates, some proteins are processed by Hsp70 and then transferred to the Hsp90 machinery. In this case, the scaffold protein Hop connects elements of the Hsp70 and Hsp90 machineries to form the "intermediate complex." The Hsp70 component dissociates and, at the same time, p23 and prolyl isomerases enter the complex. After that, the substrate is released from this "final complex." After binding to Hop, Hsp90 is able to reenter the chaperone cycle (Wegele et al., 2004). There is some evidence that Hsp90 is also able to act independently of Hsp70. Each chaperone-folding pathway can either lead to folded, functional proteins or to degradation. Neither Hsp70 nor Hsp90 acts alone. The activity of both chaperones is precisely regulated by a number of co-chaperones. Interestingly, some of the co-chaperones (e.g., Hop and CHIP) are able to interact with both Hsp70 and Hsp90. Other co-chaperones are specific for the individual chaperone machinery: Hsp70 exclusively interacts with J proteins, Hip, Hsp110, and BAG-1, whereas co-chaperone p23, a signal-transduction-related protein Cdc37/p50 (which is required for the Hsp90 substrate-specific folding activity), prolyl isomerases FKPB51, FKPB52, and Cyp40 (also known as immunophilins), prolyl isomerase-related protein XAP5, phosphatase PP5, and the Hsp90 ATPase regulator Aha1 are specific cofactors of Hsp90 (Wegele et al., 2004). Overall, the Hsp90 system is a complex machinery, the uniqueness of which is defined by its close collaboration with Hsp70 and the large number of cofactors.

1.2.2.2 Hsp90. Hsp90 is one of the most abundant proteins in unstressed cells accounting for 1-2% of total soluble cell protein (Lai *et al.*, 1984; Welch and Feramisco, 1982). In eukaryotes, cytoplasmic Hsp90 is essential for viability under all conditions. There are two genes encoding cytosolic Hsp90 homologues in mammalian cells. For example, the human Hsp90 α shows 85% sequence identity to Hsp90 β (Hickey *et al.*, 1989). ATP hydrolysis is crucial for Hsp90 function *in vivo*. However, ATP binding to Hsp90 is generally weak with a dissociation constant in the high micromolar range (Prodromou *et al.*, 1997). The ATPase activity of human Hsp90 is barely detectable, with a k_{cat} of 0.089 \pm 0.004 min⁻¹ and a K_m of 840 \pm 60 μ M (McLaughlin *et al.*, 2002). Therefore, as in the case of Hsp70, the ATPase cycle of Hsp90 is modulated by partner proteins that act in complex with Hsp90 *in vivo*.

Hsp90 is known to regulate a number of specific targets. Among the established substrates or "client proteins" of Hsp90 are transcription factors, such as steroid hormone receptors and p53, as well as some proto-oncogenic serine/threonine and tyrosine kinases, such as Raf and Src in higher eukaryotes (for reviews see Buchner (1999), Picard (2002), and Pratt and Toft (2003)).

In neurodegenerative disorders associated with protein aggregation, Hsp90 is known to regulate the heat shock response (Barral *et al.*, 2004; Klettner, 2004;

Soti *et al.*, 2005). Inhibition of Hsp90 activates heat shock factor 1 (HSF1) to induce the production of the chaperones Hsp70 and Hsp40, which promote disaggregation and protein degradation. Under nonstressed conditions, Hsp90 binds to HSF1 and maintains the transcription factor in a monomeric state (Zou *et al.*, 1998). Stress-induced inhibition of Hsp90 releases HSF1 from the Hsp90 complex, leading to its trimerization, activation, and translocation to the nucleus where it initiates a heat shock response (Zou *et al.*, 1998).

Structurally, Hsp90 is an elongated dimer (Maruya *et al.*, 1999; Richter *et al.*, 2001), which, in higher eukaryotes, exists either as α - α or β - β homodimers or as α - β heterodimers (Nemoto *et al.*, 1996; Perdew *et al.*, 1993). The quaternary structure is important for the ATPase activity and associated conformational changes. There are three major domains in Hsp90: a highly conserved N-terminal ATPase domain; a middle domain, which is potentially involved in binding of the substrate proteins; and a C-terminal dimerization domain, which is essential for Hsp90 function and provides the binding site for a subset of Hsp90 co-chaperones, containing TPR domains (Chadli *et al.*, 2000; Chen *et al.*, 1998; Maruya *et al.*, 1999; Prodromou *et al.*, 1997). In eukaryotic Hsp90s, the aminoterminal nucleotide-binding domain is connected to the remainder of the protein by a highly charged and protease-sensitive segment that is variable both in length and composition between different species and between different isoforms in the same species (Wegele *et al.*, 2004).

Because of the intrinsic conformational flexibility of the intact protein, for a long time, atomic resolution crystal structures have only been solved for individual structural domains of Hsp90. Recently, solution structure of the first nucleotide-free eukaryotic Hsp90 (apo-Hsp90) from pig brain was analyzed using the combination of small-angle X-ray scattering and single-particle cryo-electron microscopy (cryo-EM). This analysis revealed the intrinsic flexibility of the full-length eukaryotic apo-Hsp90 and showed that apo-Hsp90 exists in a conformational equilibrium between two open states, transitions between these, the fully open and the semi-open states, require large movements of the N-terminal domain and middle domain around two flexible hinge regions (Bron *et al.*, 2008). Figure 1.5a illustrates the disorder distribution within the sequence of human Hsp90 α , whereas Figure 1.5b shows the disorder status in human Hsp90 β . Data of this analysis show that in agreement with structural studies functional domains are separated by long disordered regions.

1.2.2.3 Co-chaperone p23. p23 (also known as prostaglandin E synthase 3, telomerase-binding protein p23, and progesterone receptor complex p23 in human or Sba1 in yeast) is a small protein with chaperone activity (Bose *et al.*, 1996). Similar to Hop, p23 interacts with the N-terminal ATPase domain of Hsp90 (Wegele *et al.*, 2003). This interaction is dependent on ATP binding (Prodromou *et al.*, 2000; Sullivan *et al.*, 2002; Young and Hartl, 2000) and inhibits the intrinsic ATPase activity of Hsp90 (Panaretou *et al.*, 2002). In yeast, amino-terminal dimerization of Hsp90 noticeably increases the affinity of p23 for yeast Hsp90.



Figure 1.5 The disorder distribution within the sequence of human Hsp90 α (a), and the disorder status in human Hsp90 β (b). In agreement with structural studies, functional domains are separated by long disordered regions.

The p23 interaction is counteracted by Hop, which prevents amino-terminal dimerization of yeast Hsp90 and therefore binding of p23 to yeast Hsp90 (Wegele *et al.*, 2003).

The N-terminal region of p23 contains a CS domain, which is an ~100-residue protein-protein interaction module named after CHORD-containing proteins and SGT1 (Shirasu *et al.*, 1999). The CS domain has a compact antiparallel β sandwich fold consisting of seven β -strands (Garcia-Ranea *et al.*, 2002). In the crystal structure of human p23 (Weaver *et al.*, 2000), the C-terminal tail (residues 91–160) is unresolved, which implies that it may be unstructured. This disordered tail occupies almost one half of the protein and is highly enriched in Asp and Glu residues. Although this flexible tail is not needed for the binding of p23 to Hsp90, it is necessary for optimum active chaperoning activity of p23 in assays measuring inhibition of heat-induced protein aggregation (Weaver *et al.*, 2000; Weikl *et al.*, 1999). In agreement with this structural analysis, Figure 1.6a shows a very high level of predicted intrinsic disorder in the C-terminal half of human p23.



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Figure 1.6 Predicted intrinsic disorder in Hsp90 co-chaperones and accessory proteins. Disorder distribution in human p23 co-chaperone (a) and in human FKBP52, also known as FK506-binding protein 4, HSP-binding immunophilin (HBI), 52 kDa FK506-binding protein, FKBP59, and p59 protein (b).

1.2.2.4 Prolyl Isomerases/Immunophilins. The immunophilins are ubiquitous and conserved proteins that have peptidylprolyl isomerase (PPIase) activity, suggesting that they may play a role in protein folding in the cell (Schmid, 1993). Functionally, immunophilins are divided into two classes: the cyclophilins (CsAbinding proteins) and the FKBP (FK506/rapamycin-binding proteins) (Galat, 1993). High molecular mass immunophilins possess several TPR domains and a calmodulin-binding domain in their C-terminal half (Pratt and Toft, 1997). The

binding of immunophilins to Hsp90 via TPR domains is conserved in plants and in the animal kingdom (Owens-Grillo *et al.*, 1996), suggesting that this is a basic function of the high molecular mass immunophilins.

High molecular mass prolyl isomerases/immunophilins are crucial for the effective action of the Hsp90 machinery, where together with p23 they are involved in the release of the substrate protein from the Hsp70-Hop complex, and contribute to the formation of the "final complex." In more detail, this mechanism looks as follows. The substrate protein bound to Hsp70 is brought into contact with Hsp90 via Hop. Hop and Hsp70 as parts of the "intermediate complex" are exchanged for a prolyl isomerase/immunophilin and p23 to yield the "final complex." Upon maturation from the "intermediate complex" to the "final complex," the substrate is transferred from Hsp70 to Hsp90 (Wegele *et al.*, 2003). In addition to this crucial role in the Hsp70–Hsp90 chaperone cycle, immunophilins are involved in a number of very important biological processes, for example, in hormonal activation. For example, in the absence of hormone, the glucocorticoid receptor (GR), which is a hormone-activated transcription factor that requires hormonally driven movement to its site of action within the nucleus, is found in the cytosolic fraction of cells as a mixture of complexes. The common feature of all of these complexes is that they all contain GR and Hsp90. However, each of these heterogeneous complexes contains only one molecule of either FKBP52, FKBP51, Cyp40, or PP5, which are unified by being the TPR domaincontaining members of the prolyl isomerase/immunophilin family (Davies et al., 2002).

In FKBP52, there are three globular domains followed by a C-terminal portion containing a predicted calmodulin-binding domain (Callebaut *et al.*, 1992). The N-terminal domain possesses the highest homology (49%) with the wellcharacterized low molecular mass prolyl isomerase FKBP12 (Callebaut *et al.*, 1992). This domain has PPIase activity *in vitro* (Chambraud *et al.*, 1993) and possesses the dimerization site (Wiederrecht *et al.*, 1992). Domain II, which virtually has no PPIase activity (Chambraud *et al.*, 1993), is less homologous to FKBP12 (28%) and contains a consensus nucleotide-binding sequence (Callebaut *et al.*, 1992). Domain III, deletion of which abrogates FKBP52 binding to Hsp90, comprises three TPR domains (Radanyi *et al.*, 1994). The functionality of predicted calmodulin-binding domain at the C-terminus of FKBP52 was supported by the specific retention of this protein by calmodulin-Sepharose in the presence of calcium (Massol *et al.*, 1992).

Figure 1.6b shows that human FKBP52 (also known as FK506-binding protein 4, HSP-binding immunophilin (HBI), 52-kDa FK506-binding protein, FKBP59, and p59 protein) contains a noticeable amount of disorder. In fact, both ends of the protein are highly disordered. Furthermore, although FKBP52 contains several structured domains (PPIase type 1, PPIase type 2, and three TPR domains), all of them are separated by highly flexible linkers, providing the unique functional plasticity to the protein, where each domain can act independently from its neighbors.

1.2.3 Small Heat Shock Proteins

sHsps constitute a structurally divergent family of stress proteins characterized by the presence of the α -crystallin domain, a conserved sequence of 80–100 amino acid residues (Boelens *et al.*, 1998; Carver, 1999; Derham and Harding, 1999; Kim *et al.*, 1998; van Montfort *et al.*, 2001b). The sHSPs are molecular chaperones, storing aggregation-prone proteins as folding competent intermediates and conferring enhanced stress resistance on cells by suppressing aggregation of denatured or nonfolded proteins.

The core of sHsps is the conserved α -crystallin domain, which is typically located in the middle of sHSPs, being flanked by two extensions. The α -crystallin domains of sHsps share a common structure in all members of this family consisting of a seven-stranded, IgG-like β -sandwich with topology identical to p23 (Kim et al., 1998; van Montfort et al., 2001a,b). The poorly conserved N-terminal region varies in sequence and length and influences oligomer construction and chaperone activity. The highly flexible and variable C-terminal extension stabilizes quaternary structure and enhances protein/substrate complex solubility (Carver, 1999). In human sHsps, α -crystallin domain modulates both the structural integrity and the function. This domain is similar to a major lens protein α -crystallin that is composed of two similar subunits, α A- and α B-crystallins. α -Crystallin is highly abundant in lens cells, where it comprises as much as 40% of the cytoplasmic protein and is typically assembled into a heterogeneous mixture of large complexes (Derham and Harding, 1999). Electron microscopic analysis of a 32-subunit complex of α B-crystallin reveals a micelle-like hollow globular structure with outside dimensions of \sim 190 Å and inside dimensions of \sim 100 Å (Haley et al., 1998).

The molecular mass of various sHSPs in different species ranges from 12 to 43 kDa, and even within a single species, most organisms express multiple sHsps in a cell-specific and developmentally regulated pattern (Kappe et al., 2003). For example, the number of sHsp genes in the known eukaryotic genomes ranges from 2 in yeast to 12 in Drosophila melanogaster (Michaud et al., 2002), 16 in Caenorhabditis elegans (Candido, 2002), and 19 in Arabidopsis thaliana (Scharf et al., 2001). Furthermore, Arabidopsis thaliana genome contains additional distantly related 25 genes coding for proteins that contain one or more α-crystallin domains (Scharf et al., 2001). In humans, there are 10 sHSPs, many of which are constitutively present at high levels and implicated in various diseases (Franck et al., 2004; Kappe et al., 2003). These 10 human sHsps are Hsp27/HspB1, HspB2, HspB3, αA-crystallin/HspB4, αB-crystallin/HspB5, Hsp20/HspB6, cvHsp/HspB7, H11/HspB8, HspB9, and a sperm tail protein known as outer dense fiber protein 1 (ODF1) (Kappe et al., 2003). Their genes are dispersed over nine chromosomes, suggesting their ancient origin (Kappe et al., 2003).

The sHsps occur as homo- or heteromeric complexes, comprising about 2-40 subunits. These globular complexes are often polydisperse and dynamic, readily exchanging subunits, and bind a wide range of cellular substrates. sHsps serve

mostly as "holdases," prevent the in vitro aggregation of unfolding proteins, which can be transferred to ATP-dependent chaperones, such as Hsp70, and refolded (Haslbeck and Buchner, 2002). sHSP-substrate binding capacity is known to be enhanced by structural changes that expose hydrophobic surfaces that are normally occluded in the native sHsp oligomeric structure (van Montfort et al., 2001a). Putative substrate binding sites may become available through dissociation of sHsp oligomers to dimers as a result of the dynamic equilibrium of sHsp subunits between oligomeric and suboligomeric species or through more subtle environmentally induced (e.g., high temperature) changes in sHSP tertiary structure (Haslbeck et al., 2005; van Montfort et al., 2001a). Therefore, one proposed mechanism of action of sHsps involves breaking down the large oligomer into smaller subunits, exposing hydrophobic surfaces in the α -crystallin domain, which enables binding of the unfolded substrate, followed by reassembly into large soluble complexes aided by sequence extensions (Stamler et al., 2005). sHsps protect against several cellular stressors (Arrigo et al., 2002; Latchman, 2002) and therefore their expression can be upregulated by various forms of stress (Davidson et al., 2002; Michaud et al., 2002). For example, increased levels of several human sHsps were found in neurodegenerative disorders (Krueger-Naug et al., 2002) and in certain tumors (Ciocca and Vargas-Roig, 2002).

Many of the sHsps are multifunctional proteins. For example, in addition to serving as an ATP-independent chaperone involved in protein folding, human Hsp27 (also known as HspB1) is involved in interactions with various cell structures and is implicated in architecture of the cytoskeleton, cell migration, metabolism, cell survival, growth/differentiation, mRNA stabilization, and tumor progression (Kostenko and Moens, 2009). Furthermore, a variety of stimuli induce phosphorylation of this protein at serine residues 15, 78, and 82, which is crucial for its subsequent activity (Kostenko and Moens, 2009). This functional diversity of sHsps is translated into their exceptional structural plasticity and flexibility. For example, human HspB8 (also known as Hsp22), which was shown to decrease or prevent aggregation of Huntingtin fragments and A β_{1-40} of the Dutch type, is a highly flexible protein belonging to the group of IDPs (Shemetov et al., 2008). Recently, a comprehensive search for the specific positions of a sHsp that interact directly with partially denatured substrates revealed that although all three domains of the chaperone, the N-terminal arm, the α -crystallin domain, and the C-terminal arm, are able to interact with the substrate, the N-terminal extension plays the most important role in the substrate binding. Several substrates were shown to form strong contacts with multiple residues of this region, the intrinsically disordered nature of which helps in adopting diverse geometries of interaction sites necessary for the interaction with different substrate proteins. This property of the N-terminal arm is critical for the ability of sHsps to protect efficiently many different substrates (Jaya et al., 2009). Figure 1.7 reports on the disorder status of human sHsps and shows that many of these chaperones are highly disordered.



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Figure 1.7 The distribution of PONDR VSL2 predicted intrinsic disorder in sequences of human small heat shock proteins, sHsps: Hsp27/HspB1 (a), HspB2 (b), HspB3 (c), α A-crystallin/HspB4 (d), α B-crystallin/HspB5 (e), Hsp20/HspB6 (f), cvHsp/HspB7 (g), H11/HspB8 (h), HspB9 (i), and outer dense fiber protein 1 (ODF1) (j). In each plot, the localization of the α -crystallin domain is indicated as gray area.

1.2.4 Synucleins

Synucleins belong to a family of closely related presynaptic proteins that arise from three distinct genes, described currently only in vertebrates (Clayton and George, 1999). This family includes α -synuclein, which is also known as the non-amyloid component precursor protein (NACP) or synelfin (Jakes *et al.*, 1994; Maroteaux *et al.*, 1988; Ueda *et al.*, 1993); β -synuclein, also referred to as phosphoneuro-protein 14 or PNP14 (Jakes *et al.*, 1994; Nakajo *et al.*, 1993; Tobe *et al.*, 1992); and γ -synuclein, also known as *breast cancer-specific gene 1* or BCSG1 and persyn (Buchman *et al.*, 1998a,b; Ji *et al.*, 1997; Lavedan *et al.*, 1998; Ninkina *et al.*, 1998). All three proteins belong to the family of IDPs (Uversky *et al.*, 2002a; Yamin *et al.*, 2005), with α -synuclein being one of the most well-characterized IDPs (Uversky, 2003a, 2007, 2008, 2009; Uversky and Eliezer, 2009; Uversky *et al.*, 2001).

 α -Synuclein is an abundant presynaptic brain protein, whose misfolding, aggregation, and fibrillation are implicated as critical factors in several neurodegenerative diseases. α -Synucleins from different organisms possess a high degree of sequence conservation. For example, mouse and rat α -synucleins are identical throughout the first 93 residues, whereas human and canary proteins differ from them by only two residues (Clayton and George, 1998). At least three α -synuclein isoforms are produced in humans by alternative splicing (Beyer, 2006). The best known isoform is α -synuclein-140 which is the whole and the major transcript of the protein. Two other isoforms, α -synuclein-126 and α -synuclein-112, are produced by AS resulting from the in-frame deletion of exons 3 and 5, respectively. Exon 3 localizes at the N-terminal of the protein and codes for amino acid residues 41–54, whereas exon 5 is located at the C-terminal domain of the protein, coding for residues 103–130. The whole transcript of human α -synuclein, a protein composed of 140 amino acid residues, can be divided into three regions:

- 1. Residues 1–60 form the N-terminal region. It includes the sites of three familial PD mutations and contains four 11-amino acid imperfect repeats with a highly conservative hexameric motif (KTKEGV). The N-terminal region is predicted to form amphipathic α -helices, typical of the lipid-binding domain of apolipoproteins (Clayton and George, 1998; George *et al.*, 1995).
- 2. Residues 61–95 constitute the central region and comprise the highly amyloidogenic NAC sequence (NAC stays for *Non-A* β Component of AD amyloid) (Han *et al.*, 1995; Ueda *et al.*, 1993). NAC contains three additional KTKEGV repeats and represents a second major intrinsic constituent of Alzheimer's plaques, amounting to about 10% of these inclusions (Ueda *et al.*, 1993). An 11-amino-acid segment within the central part of the NAC domain (corresponding to residues 73–83 of α -synuclein) is missing in β -synuclein.
- 3. The highly charged C-terminal region is constituted by residues 96–140. This part of α -synuclein is highly enriched in acidic residues and prolines, suggesting that it adopts a disordered conformation. Three highly conserved tyrosine residues, which are considered as a family signature of α and β -synucleins, are located in this region. This region is mostly missing in γ -synuclein.

 α -Synuclein exhibits a 40% homology with members of the 14-3-3 chaperone protein family (Ostrerova *et al.*, 1999). The 14-3-3 proteins constitute a family of protein chaperones that are particularly abundant in the brain, similar to α -synuclein. The 14-3-3 family of proteins consists of five different isoforms that share extensive sequence homology, both among the different isoforms and between similar isoforms in different species (Broadie *et al.*, 1997; Layfield *et al.*, 1996). 14-3-3 Proteins appear to be involved in diverse cellular functions, mostly

via the regulation of protein kinases (Aitken, 1995; Aitken *et al.*, 1995). They bind to ligands at sites containing phosphoserine residues. Binding of 14-3-3 to phosphorylated Raf-1 stabilizes it in an active conformation (Tzivion *et al.*, 1998). 14-3-3 binds to a phosphorylated epitope of protein kinase C ε (PKC ε) and stabilizes PKC ε in an inactive conformation that is unable to translocate to the membrane (Meller *et al.*, 1996). 14-3-3 also binds to phosphorylated death agonist BAD, a very distant BCL2 family member and a pro-apoptotic oncogene that remains inactive when sequestered in the cytosol. The interaction of 14-3-3 with BAD was shown to stabilize maintenance of BAD in a cytoplasmic localization (Zha *et al.*, 1996).

Deposition of α -synuclein has been implicated in the pathogenesis of several neurodegenerative disorders, known as synucleinopathies. Synucleinopathies share common pathologic proteinaceous lesions that are composed of aggregated α -synuclein and are deposited in the selectively vulnerable populations of neurons and glia (Galvin et al., 2001; Goedert, 1999; Spillantini and Goedert, 2000; Trojanowski and Lee, 2003). The term synucleinopathies was introduced in 1998 (i.e., just one year after the discovery of α -synuclein deposition in PD) when it was recognized that filamentous α -synuclein deposits might represent a common hallmark linking MSA with PD and DLB (Spillantini et al., 1998b). In addition to these three diseases, the current list of the synucleinopathies includes (but is not limited to) neurodegeneration with brain iron accumulation, type I (also known as adult neuroaxonal dystrophy or Hallervorden-Spatz diseases (HSD)); pure autonomic failure and several Lewy body disorders; and diffuse Lewy body disease (DLBD), the Lewy body variant of Alzheimer's disease (LBVAD) (Arawaka et al., 1998; Gai et al., 1998; Lucking and Brice, 2000; Okazaki et al., 1961; Spillantini et al., 1998a, b 1997; Takeda et al., 1998; Trojanowski et al., 1998; Wakabayashi et al., 1997, 1998). Furthermore, even before the detection of asynuclein as the major Lewy body (LB) component in PD, the peptide derived from the central hydrophobic region of this protein (residues 61-95), known as NAC, was found to represent a second major intrinsic constituent of the AD senile plaques (Han et al., 1995; Ueda et al., 1993). Intriguingly, subsequent work failed to confirm the presence of NAC in amyloid plaques (Bayer et al., 1999). Growing evidence associates the onset and progression of clinical symptoms as well as the degeneration of affected brain regions in these neurodegenerative disorders with the formation of abnormal filamentous aggregates containing α -synuclein. Therefore, it has been concluded that all aforementioned disorders are brain amyloidoses unified by pathological intracellular inclusions of aggregates having the α-synuclein protein as a key component (Galvin et al., 2001; Goedert, 1999; Lundvig et al., 2005; Spillantini et al., 1998a, b; Spillantini and Goedert, 2000; Trojanowski and Lee, 2003; Wakabayashi et al., 1997). Some key facts linking α -synuclein aggregation with the pathogenesis of different synucleinopathies are outlined below. It is believed that understanding why α -synuclein pathology develops in these apparently unrelated conditions may shed light on the mechanisms operating in different synucleinopathies (Goedert, 2001).

It has recently been established that in addition to the traditional α -synucleincontaining LBs and LNs, the development of PD and DLB is accompanied by the appearance of novel α -, β -, and γ -synuclein-positive lesions at the axon terminals of hippocampus (Galvin et al., 1999). These pathological vesicular-like lesions located at the presynaptic axon terminals in the hippocampal dentate, hilar, and CA2/3 regions have been co-stained by antibodies to α - and β -synucleins, whereas antibodies to γ -synuclein detect previously unrecognized axonal spheroid-like inclusions in the hippocampal dentate molecular layer (Galvin et al., 1999). This broadens the concept of neurodegenerative "synucleinopathies" by implicating β - and γ -synucleins, in addition to α -synuclein, in the onset/progression of these two diseases. Additionally, abnormal expression of γ -synuclein has recently been reported in some breast tumors (Ninkina et al., 1998). Using Northern blots and in situ hybridization, it has been shown that a high percentage of malignant breast tumors, but not benign breast tumors or normal breast tissue, express y-synuclein mRNA (Ninkina et al., 1998). In addition, a direct link between y-synuclein overexpression and increased invasiveness of breast tumor cells has been demonstrated (Ninkina et al., 1999).

Human β -synuclein is a 134-amino acid neuronal protein showing 78% identity to α -synuclein. The α - and β -synucleins share a conserved C-terminus with three identically placed tyrosine residues. However, β -synuclein is missing 11 residues within the specific NAC region (Clayton and George, 1998; Lucking and Brice, 2000). The activity of β -synuclein may be regulated by phosphorylation (Nakajo *et al.*, 1993). This protein, like α -synuclein, is expressed predominantly in the brain; however, in contrast to α -synuclein, β -synuclein is distributed more uniformly throughout the brain (Nakajo *et al.*, 1994; Shibayama-Imazu *et al.*, 1993). Besides the central nervous system, β -synuclein was also found in Sertoli cells of the testis (Nakajo *et al.*, 1996; Shibayama-Imazu *et al.*, 1998), whereas α -synuclein was found in platelets (Hashimoto *et al.*, 1997).

The third member of the human synuclein family is the 127-aa γ -synuclein, which shares 60% similarity with α -synuclein at the amino acid sequence level (Clayton and George, 1998; Lucking and Brice, 2000). This protein specifically lacks the tyrosine-rich C-terminal signature of α - and β -synucleins (Clayton and George, 1998). γ -Synuclein is abundant in spinal cord and sensory ganglia (Buchman *et al.*, 1998a, b). Interestingly, this protein is more widely distributed within the neuronal cytoplasm than α - and β -synucleins, being present throughout the cell body and axons (Buchman *et al.*, 1998b). It was also found in metastatic breast cancer tissue (Ninkina *et al.*, 1998) and epidermis (Ninkina *et al.*, 1999).

Despite the facts that α -synuclein was estimated to account for as much as 1% of the total protein in soluble cytosolic brain fractions (Iwai *et al.*, 1995) and that it is assumed to play a crucial role in the pathogenesis of several neurodegenerative disorders, the precise function of this protein remains mainly elusive. α -Synuclein is expressed in a number of neuronal and non-neuronal cell types including cortical neurons, dopaminergic neurons, noradrenergic neurons, endothelial cells, and platelets (Abeliovich *et al.*, 2000; Hashimoto *et al.*, 1997;

Li et al., 2002; Tamo et al., 2002). Interestingly, torpedo synuclein was reported to localize within the nucleus and presynaptic nerve terminals (Maroteaux et al., 1988); however, most subsequent studies have shown α -synuclein localization only within nerve terminals in the central nervous system (Clayton and George, 1998, 1999; Lavedan, 1998). Although the precise function of α -synuclein remains unknown, this localization, in addition to the close association of this protein with vesicular structures, has led to the hypothesis that it may regulate vesicular release and/or turnover and synaptic function in the central nervous system (Clayton and George, 1998, 1999; Davidson et al., 1998; Lavedan, 1998; Ueda *et al.*, 1993). In agreement with this hypothesis, mice lacking α -synuclein, being superficially normal, exhibited alterations in transmitter release from dopaminergic terminals in striatum, following paired electrical stimulation and in locomotor responses after amphetamine administration (Abeliovich et al., 2000). Additional observations suggest that α -synuclein may play a role in neuronal plasticity responses because its avian homolog synelphin is upregulated in zebra finch brain at a critical period of song learning (George et al., 1995), and rat synuclein-1 is upregulated during brain development (Hsu et al., 1998; Petersen et al., 1999) and in cultured neonatal sympathetic neurons after nerve growth factor treatment (Stefanis et al., 2001). a-Synuclein was shown to act as a high-affinity inhibitor of phospholipase D2, which hydrolyzes phosphatidylcholine to phosphatidic acid and may be involved in vesicle trafficking in the secretory pathway (Chen et al., 1997; Jenco et al., 1998). Overall, functions ascribed to α -synuclein include binding fatty acids and physiological regulation of certain enzymes, transporters, and neurotransmitter vesicles, as well as roles in neuronal survival (Dev et al., 2003).

It has been shown that α -synuclein can act as a molecular chaperone (Chandra *et al.*, 2005), cellular levels of which in both substantia nigra and frontal cortex were shown to be significantly increased as a response to the toxic insult (Manning-Bog *et al.*, 2002). These toxicant-induced changes in the expression of α -synuclein were characterized by a very peculiar time course where levels of the protein in the mice brain were consistently enhanced at two days after paraquat administrations and returned to basal control values within seven days posttreatment (Manning-Bog *et al.*, 2002). A similar time course of α -synuclein upregulation has also been reported for mice treated with the parkinsonism-inducing neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Vila *et al.*, 2000). These findings suggested that α -synuclein plays a role in overcoming the consequences of the toxic insult. Furthermore, overexpression of α -synuclein was shown to actually delay cell death caused by toxic agents and protect cells against the apoptotic stimuli (da Costa *et al.*, 2000; Lee *et al.*, 2001).

Methionine oxidation was proposed to play a role in the potential α -synuclein function as a chaperone. In fact, since the addition of methionine-oxidized α -synuclein inhibited fibrillation of the nonoxidized form, it was suggested that the methionine residues in α -synuclein may be used by the cells as a natural scavenger of ROS, and therefore, α -synuclein can serve as a redox chaperone (Uversky *et al.*, 2002b). This hypothesis was based on the facts that

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(i) methionine can react with essentially all of the known oxidants found in normal and pathological tissues; (ii) α -synuclein is a very abundant brain protein; (iii) the concentration of α -synuclein can increase significantly as a result of the neuronal response to toxic insult (Manning-Bog *et al.*, 2002); and (iv) methionine sulfoxide residues in proteins can be cycled back to their native methionines by methionine sulfoxide reductase (Levine *et al.*, 1996), a process that might protect other functionally essential residues from oxidative damage (Reddy and Bhagyalakshmi, 1994). However, the antifibrillation role of the methionine-oxidized α -synuclein was strongly compromised in the presence of certain heavy metals, such as lead, aluminum, zinc, titanium, and others (Yamin *et al.*, 2003). Therefore, in the presence of the enhanced concentrations of such industrial pollutants, toxic insult-induced upregulation of α -synuclein may no longer play a protective role; rather, it may represent a risk factor, leading to metal-triggered fibrillation of the methionine-oxidized protein (Yamin *et al.*, 2003).

Comprehensive structural analysis of human α -, β -, and γ -synucleins revealed that these three proteins are almost completely unfolded under the physiological conditions *in vitro* (Uversky *et al.*, 2002a). Figure 1.8 supports this experimental data-based conclusion and shows that all three human synucleins are predicted to be completely disordered.

1.3 THE ACTION MECHANISMS OF INTRINSICALLY DISORDERED CHAPERONES

On the basis of their molecular mechanism of action, chaperones have been divided into several functional subclasses: the "(un)folding" chaperones that utilize the ATP-dependent conformational changes to promote unfolding and subsequent refolding of their substrates; the "holding" chaperones that hold partially folded substrates in the folding-competent state, preventing their aggregation while waiting for the available "folding" chaperones; the "disaggregating" chaperones that are responsible for the solubilization of aggregated proteins; the "folding rate-limiting steps; and the "redox" chaperones that help prevent the consequences of the oxidative damage. Illustrative members of all these chaperone classes were discussed in this chapter to show that all of them clearly belong to the family of IDPs, being either completely disordered or containing functionally important long disordered regions. Some potential modes of the intrinsic disorder involvement in the function of some of these proteins are briefly considered below.

1.3.1 Intrinsically Disordered Holding Chaperones

Many proteins in their misfolded or partially (un)folded conformation(s) are sticky and therefore possess increased propensity to aggregate. The efficiency of an aggregation process depends dramatically on protein concentration. Therefore,



Figure 1.8 Intrinsic disorder in human α - (a), β - (b), and γ -synucleins (c) as evaluated by PONDR VSL2.

the major task of holding chaperones or holdases is to bind misfolded or partially (un)folded substrates, to decrease the pool of free molecules available for nonproductive and potentially toxic aggregation, and to hold misfolded/aggregated proteins until they can be disaggregated and refolded by the ATP-dependent chaperones. As mentioned above, sHsps, which normally exist as oligomers that are polydisperse and change size and organization on exposure to stress and when interacting with substrate (Basha *et al.*, 2004b; Ehrnsperger *et al.*, 1997; Horwitz, 1992; Lee *et al.*, 1997; Studer and Narberhaus, 2000), serve as holding chaperones. Three possible modes of chaperone action have been suggested for sHsps:

1. The large oligomeric form remains intact as it binds the substrate on its surface (Kim *et al.*, 2003);

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- 2. The large oligomer breaks down into smaller subunits (that may be monomers, dimers, or larger assemblies), which exposes hydrophobic surfaces enabling binding of unfolded substrate, and subsequently reassembles into large soluble complexes that are then handled by ATP-dependent refolding machinery (Haslbeck *et al.*, 1999; Stamler *et al.*, 2005; van Montfort *et al.*, 2001a); and
- 3. sHsp molecules are intercalated into large insoluble protein aggregates, which enables subsequent disaggregation and refolding by the ATP-dependent refolding machinery (Basha *et al.*, 2004a; Cashikar *et al.*, 2005; Haslbeck *et al.*, 2005; Mogk *et al.*, 2003).

It is also possible that all three mechanisms are realized in nature and that the holding chaperones use different approaches to work with the different targets. Irrespective of the mechanism of action, intrinsic disorder is crucial for holding chaperones. Model substrates protected *in vitro* as well as proteins found associated with sHsps *in vivo* include proteins of a wide range of molecular masses, pI values, and structures, with no obvious common characteristics (Basha *et al.*, 2004a; Haslbeck *et al.*, 2004a). This astonishing promiscuity of sHsps and their ability to interact with very different targets "frozen" at different folding stages rely on the flexibility of the chaperones' binding sites. In fact, the intrinsically disordered nature of these binding sites helps them to accommodate different substrate proteins by adopting structurally diverse binding platforms (Jaya *et al.*, 2009). The fact that the surface of a holding chaperone possesses multiple substrate-binding sites determines the peculiar interaction mechanism where sHsps bind coils and secondary structural elements by wrapping them around the α -crystallin domain (Stamler *et al.*, 2005).

Furthermore, intrinsically disordered N- and C-terminal arms of sHsps play a number of roles important for the sHsp structure and function. They are involved in the regulation of the oligomeric state of these chaperones, stabilize their quaternary structure, regulate chaperone activity, and enhance solubility of the protein/substrate complex (Carver, 1999). For example, the evolutionarily variable N-terminal arms of sHSP subunits, which are often unresolved in the crystal structures of sHsps (Sharma et al., 1997; van Montfort et al., 2001b) and are disordered in cryo-EM images of α -crystallins (Haley et al., 2000), are essential for interaction with substrates, and sHSPs with an N-terminal arm that is naturally short or truncated by mutagenesis most often lack chaperone function (Fu et al., 2005; Haslbeck et al., 2004b; Leroux et al., 1997; Stromer et al., 2004). Similarly, the C-terminal arms of sHsps, which protrude from the domain core of the molecule, are flexible and solvent exposed (Carver et al., 1992, 1995). These highly flexible C-terminal extensions represent a conserved feature of mammalian sHsps (Caspers et al., 1995). Although they share no sequence similarity, the C-terminal arms of the mammalian sHsps have several common characteristics, being polar, having no ordered structure, and being conformationally flexible. Importantly, these arms are not involved in direct binding to the substrate. However, they act as polar solubilizing

agents for the relatively hydrophobic sHsp proteins and the sHsp-substrate complexes (Lindner et al., 2000). If the polarity and flexibility of the extensions are disrupted, as in the hydrophobic mutant of α A-crystallin, the stability and chaperone activity of the protein are reduced significantly (Smulders et al., 1996). Because of the flexibility of the C-terminal arms of the mammalian sHsps, their solubilizing function was suggested to arise from an entropic contribution to the free energy of the solution state (Lindner *et al.*, 2000). These extensions were also proposed to serve as entropic "spacers," which prevent sHsp-substrate complexes from aggregation (Lindner et al., 2000). In other words, disordered segments may act via the entropic exclusion effect, a long-range repulsive force, which prevents molecules from approaching each other (Tompa and Csermely, 2004). Therefore, similar to the disordered regions of microtubule-associated proteins (Mukhopadhyay and Hoh, 2001), neurofilaments (Brown and Hoh, 1997), and nucleoporins (Rout et al., 2000), highly flexible extensions of holding chaperones work as entropic brushes that, because of the thermally driven motion, maintain spacing between the chaperone oligomers.

1.3.2 Intrinsically Disordered (Un)Folding Chaperones

Kinetically trapped misfolded substrates are stuck in a local conformational energy minimum. Chaperones were proposed to assist folding by randomly disrupting the misformed bonds via repeated cycles of binding and release, allowing the substrate to resume search in the conformational space toward the global energy minimum (Tompa and Csermely, 2004). Numerous data indicate that ATP-dependent molecular chaperones use free energy from ATP binding and/or hydrolysis to minimize the concentrations of misfolded and nonproductively aggregated proteins in the cell. One of the models of such chaperone action is that they serve as unfoldases that use free energy from ATP binding and/or hydrolysis to unfold misfolded proteins to yield productive folding intermediates (Hubbard and Sander, 1991; Rothman, 1989; Rothman and Kornberg, 1986; Slepenkov and Witt, 2002b). These reestablished productive folding intermediates can fold spontaneously to the native state. In this mechanism, ATP-dependent chaperones (e.g., Hsp70 proteins) are able to lower the activation energy barrier for the transitions from the misfolded species to the productive intermediates without changing the microscopic rate constant for the folding reaction. Acting as an unfoldase, ATP-dependent chaperones reverse the nonproductive reactions leading to the misfolded species, and therefore keep the polypeptide chain in a folding-competent state (Slepenkov and Witt, 2002b). The unfoldase activity was studied in detail for several members of the Hsp70 family, such as DnaK (Slepenkov and Witt, 2002b); the mitochondrial heat shock protein, mtHsp70, which was shown to unfold preproteins in an ATP-dependent mechanism before their import into mitochondria (Voisine et al., 1999); and GroEL (Shtilerman et al., 1999). The critical prerequisites for the ATP-driven chaperone to serve as an unfoldase mediating proper unfolding/refolding of many different proteins in the cell are (Sharma et al., 2009):

- 1. the propensity to specifically bind misfolded substrates with a higher affinity than their natively refolded products;
- 2. the capability to recruit the energy of ATP hydrolysis to favor unfolding of the bound misfolded substrate;
- 3. the ability to dissociate timely from the newly unfolded product, allowing the latter to refold spontaneously into a low-affinity chaperone product.

For GroEL and several other ATP-dependent chaperones, the proposed mechanism of action involves active unfolding of misfolded proteins-substrates. Importantly, to serve as an unfoldase in protein homeostasis, these ATP-dependent chaperones must collaborate with co-chaperones: GroEL works as an unfoldase in the complex with GroES, whereas Hsp70s are known to work with a J-domain co-chaperone and an NEF.

There are three potential mechanisms for the unfoldase activity of chaperones: entropic pulling model, mechanical unfolding via forcible stretching, and the entropy transfer model. The peculiarities of these mechanisms are briefly outlined below.

In Hsp70s, which consist of an N-terminal ATPase domain and a C-terminal substrate-binding domain also containing a specific lid, hydrolysis of ATP and ADP/ATP exchange in the nucleotide-binding domain control the functional properties of the substrate-binding domain by allosteric cross talk. Hsp70 interacts transiently with short extended peptide segments of the substrate made of about seven non-bulky hydrophobic residues, ideally flanked by positive charges (Rudiger *et al.*, 1997b). ATP-liganded Hsp70 exhibits low affinity for misfolded substrates and fast rates of substrate binding and release (Palleros *et al.*, 1993; Schmid *et al.*, 1994). In contrast, ADP·Hsp70 is characterized by a 100-fold higher affinity for substrate and by a very slow rate of substrate release (Mayer *et al.*, 1999; Siegenthaler and Christen, 2006). During the chaperone cycle, Hsp70s alternate between the low and the high affinity state, under the control of their co-chaperones.

Binding of Hsp70 to misfolded protein accelerates ATP hydrolysis by chaperone up to two orders of magnitude (Han and Christen, 2003; Laufen *et al.*, 1999). This substrate effect is further amplified by the J-domain-containing Hsp40 cochaperones (Laufen *et al.*, 1999). For example, DnaJ stimulates ATP hydrolysis in the substrate-bound ATP·DnaK molecules and thus promotes the formation of considerably more stable [ADP·DnaK·substrate] complexes. These long-lived chaperone–substrate complexes then act as entropic pulling species: the dangling bound chaperone molecule actively unfolds the misfolded regions that flank the chaperone-binding sites in the substrate (Sharma *et al.*, 2009) (De Los Rios *et al.*, 2006; Goloubinoff and De Los Rios, 2007). GrpE accelerates the release of ADP and rebinding of ATP, triggering the "unlocking" of DnaK from its substrate. The unlocked chaperone may dissociate from an unfolded peptide loop, which may spontaneously refold into a more native structure. The final result of this cycle is the productive transient unfolding of a stably misfolded polypeptide region.

GroEL binds nonnative proteins by means of a ring of hydrophobic residues that line the entrance to the central cavity of its heptameric ring (Braig et al., 1994; Fenton et al., 1994). When GroEL, ATP, and the GroES co-chaperonin come together, massive structure changes double the GroEL cavity volume and occlude its hydrophobic binding surface (Roseman et al., 1996; Xu et al., 1997). In fact, before the ATP and GroES that bind the binding sites of GroEL are located 25 Å from each other, whereas on addition of ATP and GroES, the apical domain of each GroEL subunit twists upward and outward so that the binding sites move apart to a position 33 Å from one another. As a result, neighboring binding sites move apart by 8 Å and nonneighboring sites by larger increments, up to 20 Å. These large-scale movements provide the means for the mechanical unfolding of the misfolded substrate protein which, being tethered to these sites, will be forcibly stretched and partially unfolded (Lorimer, 1997; Xu and Sigler, 1998). These mechanical unfolding of the misfolded substrate protein relieves it from the misfolded state, thus restoring its capability for normal folding. Incompletely folded proteins undergo further iterations until they achieve the native state (Shtilerman et al., 1999; Xu and Sigler, 1998).

Similar to the holdases discussed above, intrinsic disorder and flexibility of binding sites of ATP-dependent unfoldases add unique versatility to the recognition process since such regions can bind several different partners, enabling an enhanced speed of interaction and uncoupling specificity from binding strength (Tompa and Csermely, 2004). For example, the intrinsic disorder in the C-terminal tail of GroEL was proposed to be necessary for the ability of this chaperone to bind a wide range of unrelated, misfolded substrates (Braig *et al.*, 1994).

Another potential role of intrinsic disorder in the unfol dase activity of chaperones is described by a so-called entropy transfer model (Tompa and Csermely, 2004): the ability of disordered segments of chaperone for rapid and transient binding of the substrate and the subsequent local ordering may "pay" the thermodynamic cost of local substrate unfolding. In other words, the entropy transfer model implies the ordering of the chaperone with a concomitant unfolding of the substrate. Here, the binding-induced folding of a disordered, nonspecific binding segment of a chaperone may promote the local unfolding of the misfolded segment, and the energy required for the local unfolding of the substrate may be covered by the binding and folding of the chaperone (Tompa and Csermely, 2004).

1.3.3 Intrinsically Disordered Disaggregating Chaperones

Misfolded and partially folded proteins are often trapped in oligomeric/aggregated forms since these nonproductive aggregated states are energetically favorable and, therefore, the half-times for the return to folding-competent intermediates are typically very long. The stability of such aggregates of misfolded polypeptides is attributed to the formation of numerous small hydrophobic surfaces with increased propensity to form β -sheet-enriched structures, which tend to cooperatively associate with one another to achieve less exposure to water, thus maintaining the misfolded polypeptides tightly entangled (Sharma *et al.*,

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2009). This highly cooperative nature of intermolecular interactions between the misfolded polypeptide chains restrains the local random molecular motions which, being allowed, would eventually release individual misfolded polypeptides from the aggregate, thus giving them a chance for their native spontaneous refolding. Since aggregated proteins are potentially toxic and since the chance of their spontaneous clearance is very low, nature has elaborated a complex protective system, a network of chaperones, which can recognize misfolded proteins, prevent their aggregation, unfold misfolded regions, and disaggregate preformed aggregates. Both holding and unfolding chaperones can disaggregate nonproductive aggregates. In the cytoplasm of animal cells, unfolding and disaggregation may be achieved by Hsp70/Hsp40/NEF alone. In lower organisms, a special and very powerful disaggregating machinery has evolved. For example, yeast heat-shock protein 104 (Hsp104), plant Hsp101, and their bacterial homolog caseinolytic peptidase B (ClpB) are molecular chaperones that have the ability to solubilize almost any protein that becomes aggregated after severe stress. Although these chaperones are not found in humans, Hsp104 is of considerable interest because of its ability to dissolve numerous types of aggregates (see Chapter 7).

Unfoldases can disaggregate proteins using entropic pulling, mechanical unfolding via forced stretching, and the entropy transfer mechanisms. For example, as discussed in a recent review (Sharma et al., 2009), the energy of ATP hydrolysis serves to "lock" Hsp70 onto a loop at the surface of stable protein aggregates. Multiple ADP-liganded Hsp70 molecules tightly attached to loops of the same aggregated substrate polypeptide cooperate in applying stretching forces by entropic pulling (De Los Rios et al., 2006). A single Hsp70, or more effectively, several Hsp70 molecules, locked to loops in substrate polypeptide chains, recruit random Brownian motions to pull apart aggregated proteins and thereby distend the loop segments caught up in aggregates. The gain in entropy resulting from the increased motility of the Hsp70-loop complexes diffusing away from the aggregate may therefore overcome the aggregate-stabilizing energy (Hubbard and Sander, 1991; Rothman, 1989). In the entropy transfer mechanism, binding and folding of a disordered chaperone fragment can induce local unfolding and local disaggregation of the misfolded segment from the tightly packed aggregate. Even "passive" holdases can cause disaggregation via the entropic exclusion effects of their disordered extensions. Here, interaction of sHsp molecules with aggregated proteins may prevent and minimize intermolecular hydrophobic interactions among the aggregating polypeptides. Then, the chaperone-bound polypeptides can be "brushed away" from the aggregated species because of the entropic brush activity of the highly flexible extensions of holding chaperones that are in the constant thermally driven motion maintaining spacing between the chaperone oligomers.

1.4 CONCLUDING REMARKS

It is difficult to overestimate the role of protein chaperones in protein homeostasis. These guardians of the cell are intimately involved at all the stages of

the protein's life, starting from its birth (chaperones are awaiting the newly synthesized polypeptides exiting the ribosome and help them avoid misfolding and aggregation before the spontaneous folding into a native structure), through the maturation and productive adulthood (chaperones help in assembly of multichain complexes and in protein translocation through membranes), in the norm and in the stress (many types of stress and toxic insults can induce transient unfolding of proteins which can then misfold and aggregate, unless prevented by chaperones), and till the death (chaperones are involved in various protein's clearance mechanisms). Chaperones help proteins to fold, prevent them from misfolding and aggregation, work with misfolded and aggregated species to promote their disaggregation, productive folding, or degradation. Obviously, failure of this protective system results in the appearance of proteotoxic species that will eventually induce a strong inflammatory response, apoptosis, and tissue loss (Hinault *et al.*, 2006).

As shown in this chapter, intrinsic disorder plays a number of important roles in the action of protein chaperones. IDRs determine the promiscuity of chaperones, acting as pliable molecular recognition elements. They help to fold misfolded chains, participate in disaggregation and local unfolding of aggregated and misfolded species, and increase the solubility and foldability of proteins. The abundance of IDRs and the versatility of their functions are crucial for the success of protein chaperones.

ACKNOWLEDGMENT

This work was supported in part by the grants R01 LM007 688-01A1 and GM071 714-01A2 from the National Institute of Health, the grant EF 0 849 803 from the National Science Foundation, and the Program of the Russian Academy of Sciences for the "Molecular and Cellular Biology." We gratefully acknowledge the support of the IUPUI Signature Centers Initiative.

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