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Folding and Aggregation of Cu, Zn-Superoxide Dismutase

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1. Introduction

1.1 ALS and SOD1

In 1993, a genetic link was established between amyotrophic lateral sclerosis (ALS) and mutant forms of Cu,Zn superoxide dismutase (SOD1) (Deng et al. 1993; Rosen et al. 1993), an antioxidant enzyme that catalyzes the dismutation of the damaging free radical superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) and diatomic oxygen (O_2) via cyclic reduction and oxidation of a protein-bound Cu ion (Valentine et al. 2005). Today, over 150, predominantly missense mutations have been identified at ~75 sites spread throughout the protein (http://alsod.iop.kcl.ac.uk/). SOD1 mutations are found in ~15-20% of inherited or familial ALS (fALS) cases and in a small percentage of sporadic ALS (sALS) cases (Rosen et al. 1993; Kato et al. 2000; Liu et al. 2009; Forsberg et al. 2011). fALS accounts for ~10% of all ALS cases and so SOD1 mutations comprise ~1.5-2% of all ALS cases, but nevertheless represent a major known cause of the disease. The clinical symptoms of fALS and sALS are similar, yet fALS patients with SOD1 mutations have an earlier age of disease onset than sALS (by ~10 years) (Wijesekera and Leigh 2009). Furthermore, while the age of disease onset has not been identified as statistically different between different SOD1 mutations, disease duration for each mutation is often different, ranging from shorter (e.g. ~1 year for A4V, the most common mutation in North America) than the typical 3-5 years to longer (e.g. ~18 years for H46R) (Cudkowicz et al. 1997; Valentine et al. 2005; Wang et al. 2008). In humans and murine models of ALS, mutations in the gene encoding SOD1 are typically autosomal dominant and are associated with a toxic gain of function. Despite extensive research, the molecular basis for mutant SOD1 toxicity remains unclear (Valentine et al. 2005; Boillee et al. 2006; Ilieva et al. 2009). Extensive research has been conducted on SOD1-linked fALS, as understanding and treatment of this disease may be relevant to ALS in general. While ALS patients share many clinical symptoms, numerous genes have been linked to ALS, and there is evidence for differences in pathology related to both genetic and environmental factors; hence, ALS is a syndrome and not a single disease with unique pathology (Cozzolino et al. 2008).

Currently, there are two prevailing hypotheses for the toxic gain of SOD1 function that is observed in ALS: 1) new toxic enzymatic activity, and 2) protein misfolding resulting in formation of toxic aggregates (Valentine et al. 2005; Andersen 2006; Pasinelli and Brown 2006; Cozzolino et al. 2008; Turner and Talbot 2008). Since toxic enzymatic activity can damage the protein and cause aggregation, and conversely aggregation may result in toxic activity, these two hypotheses are not mutually exclusive. Theories involving gain of toxic activity involve altered metal binding by SOD1, resulting in the generation of reactive oxygen species, such as damaging hydroxyl and peroxynitrite radicals (Kurahashi et al. 2001; Alvarez et al. 2004). Alternatively, there is extensive evidence that ALS belongs to a growing group of protein misfolding diseases (Valentine et al. 2005; Chiti and Dobson 2006; Turner and Talbot 2008; Chiti and Dobson 2009; Deng et al. 2011). Protein inclusions, or aggregates, observed in the motor neurons and glial cells stain immunopositive for SOD1 in SOD1-linked fALS and some sALS patients (Kato et al. 2000; Liu et al. 2009; Forsberg et al. 2011) and are observed in mutant SOD1 animal models of ALS (Bruijn et al. 1998; Johnston et al. 2000). Thus, a major hypothesis in the field of ALS research is that SOD1 mutations decrease protein stability, alter protein folding and metal binding, and/or cause changes in other biophysical properties of the protein, resulting in an increased propensity of mutant SOD1 to form neurotoxic aggregates (Valentine et al. 2005).

Many reviews have summarized extensive investigations into the role of SOD1 in ALS, including *in vivo* mutant SOD1 models of ALS pathogenesis and their clinical implications (Bruijn et al. 2004; Boillee et al. 2006; Mitchell and Borasio 2007; Cozzolino et al. 2008), the numerous genetic elements and complex disease etiology associated with sALS and fALS (Boillee et al. 2006; Vucic and Kiernan 2009; Bastos et al. 2011), the various ALS rodent models used to study the underlying genetics and cause of motor neuron death in ALS (Van Den Bosch 2011), and the biophysical properties of mutant SOD1 in relation to possible disease mechanisms (Valentine et al. 2005). In this chapter we review recent research characterizing the stability, folding and misfolding, and the physical characteristics and mechanisms governing aggregation of mutant SOD1 *in vitro*. We describe in detail studies that reflect our own research and interests, but also include references to related work, to which we refer the interested reader. We will first review the general principles of protein stability and aggregation, which are pertinent to protein conformational diseases in general. Following this overview, we examine recent research that has characterized folding and aggregation of SOD1 and the relevance of this work to ALS.

1.2 Characteristics of protein aggregation

Protein aggregation is a common phenomenon observed in both normal and abnormal physiological processes, and has been studied extensively for more than 30 years (Chiti and Dobson 2006). While protein association reactions are highly regulated and essential for cellular function, unregulated protein association causes a wide range of diseases, such as sickle-cell anaemia, serpinopathies, and, in particular, many neurodegenerative diseases including prion, Parkinson's, Alzheimer's, and Huntington's diseases (Chiti and Dobson 2006; Eisenberg et al. 2006; Chiti and Dobson 2009). These protein misfolding diseases are characterized by the formation of insoluble proteinacious deposits (aggregates) (Chiti and Dobson 2006), and the mechanisms and biological effects of aggregation in different diseases are an area of active research. In some cases, toxicity may be caused by large protein aggregates; however, smaller oligomeric protein species are generally considered more

neurotoxic (Caughey and Lansbury 2003). The harmful nature of these oligomers compared to larger protein aggregates may be due to their lower stability, higher degree of solvent accessible surface area, and an increased tendency to form non-native associations with essential cellular components (Bucciantini et al. 2002; Knowles et al. 2007). For example, aggregates of many disease-associated proteins, including mutant SOD1, have been found to interact with the ubiquitin-proteasome system (Mouradian 2002; Sakamoto 2002; Urushitani et al. 2002; Valentine et al. 2005), folding chaperones (Bruening et al. 1999; Wyttenbach et al. 2000; Shinder et al. 2001; Okado-Matsumoto and Fridovich 2002), and the outer mitochondrial membrane (Vande Velde et al. 2008). These cellular components play central roles in regulating many critical cellular events ranging from cell division to apoptosis, and their impairment may represent common mechanisms by which aggregates of different proteins can cause cellular dysregulation and cell death (Hol and Scheper 2008; Gidalevitz et al. 2010). Many factors are involved in modulating protein aggregation, and are surveyed in the following sections.

1.2.1 Protein folding, stability and aggregation

Globular protein folding begins on the ribosome, as newly synthesized, unstructured polypeptide chains start to make favourable intramolecular contacts (Dobson 2004). As it further folds into its mature, native state, a protein may populate multiple conformational or intermediate states, and undergo various co- and post-translational modifications (refer to Figure 1). The rate determining step of folding involves overcoming the major energetic barrier to folding by forming a transition state complex prior to attaining the native state. In more complex cases, protein folding can involve more than one energetic barrier (Dobson 2004). Other proteins are unable to adopt a stable, well folded structure and exist as an ensemble of fluctuating, poorly structured conformations (Uversky and Dunker 2010). Thermodynamic protein stability is defined as the difference in energy between the denatured, unfolded state and the native, folded state. If there is a large separation in energy between the unfolded and folded states, the protein has high global thermodynamic stability. Stability can also be assessed by the rate of native protein unfolding, which determines how long the polypeptide remains in the folded state. This is referred to as kinetic stability, defined as the difference in energy between the folded conformation and the transition state. The closer these species are in energy, the higher the rate of unfolding and the lower the kinetic stability (Figure 1A).

In general, both thermodynamic and kinetic destabilization of proteins by chemical modifications or by mutations favours global protein unfolding and exposure of the hydrophobic groups that are normally buried in the protein core. This can promote the formation of non-native intermolecular contacts between proteins and the formation of aggregates. However, even subtle decreases in global protein stability are often accompanied by local destabilization and recent investigations have provided evidence for aggregate formation from native-like species (N*, Figure 1) that have undergone much more restricted unfolding (Nelson and Eisenberg 2006; Chiti and Dobson 2009). Examples of aggregate formation from native-like states include various proteins associated with disease such as mutant lysozyme (Chiti and Dobson 2009), β2-microglobulin (Chiti and Dobson 2009), and SOD1 (Hwang et al. 2010). The propensity of a given globular protein to aggregate depends on how energetically feasible it is for the protein to access locally, partially or fully unfolded aggregation-prone state(s). Protein folding generally occurs in a

cooperative fashion with minimal formation of partially folded species and this cooperativity generates a sufficiently large energy barrier between the unfolded and folded states, which decreases the likelihood of unfolding and aggregation (Dobson 1999; Dobson 2004; Tartaglia et al. 2008).

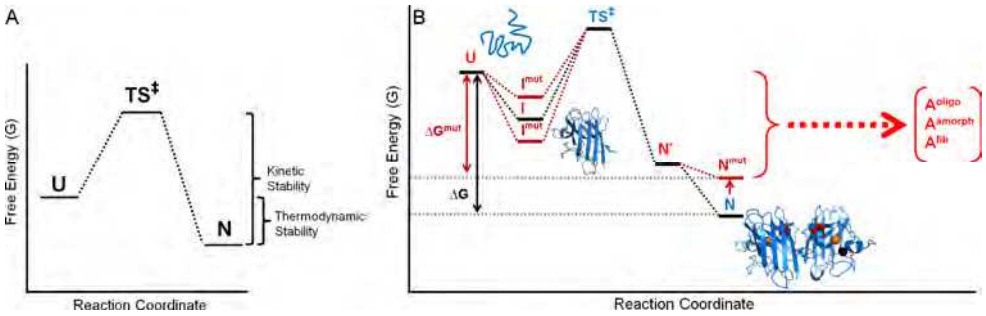

Fig. 1. Protein stability, misfolding and aggregation.
In panel A, the difference between thermodynamic and kinetic stability is shown. U, TS‡, and N refer to the unfolded, transition and native states of the protein, respectively. Refer to the main text for further explanation. In panel B, the effects of native state (N) destabilization by mutation on the population of locally unfolded, native-like (N*), partially folded intermediate (I) and fully unfolded (U) states is shown. Aggregation may occur from N*, I or U, and the morphology of the aggregates formed may depend on the conformation of the protein prior to aggregation. Mutations that destabilize N, decrease the energy difference between the N and the more unfolded states (N*, I or U), and thereby promote aggregation. Note that destabilization of N, does not necessarily imply destabilization of I. Mutations that destabilize N, may stabilize or destabilize I, resulting in a large increase or decrease, respectively, in the population of I compared to levels observed in the native folding pathway. Panel B was adapted from (Chiti and Dobson 2009).

1.2.2 Factors that modulate aggregation of polypeptides

In addition to protein stability and structure, many other factors, such as physicochemical properties of amino acids within a protein sequence and solution conditions, can affect protein aggregation. Hydrophobicity, β-sheet propensity, and charge of a polypeptide sequence have been shown to modulate the formation of amyloid aggregates (refer to section 1.2.3) by unfolded proteins (Chiti et al. 2003). Interestingly, these properties are also important for facilitating correct protein folding, suggesting that while similar forces contribute to both processes, different key residues are involved in forming the initial contacts that drive native protein folding and aggregation (Jahn and Radford 2008). In many cases, the overall aggregation propensity of a protein increases if the primary sequence contains short stretches of amino acids with properties that favour aggregation, for example, low net charge, extensive hydrophobicity, and/or a tendency to form a β-sheet over an α-helix (Tartaglia et al. 2008). Interestingly, many fALS-associated SOD1 mutations decrease the net charge of the protein, which may promote aggregation and explain why certain mutations give rise to a shorter disease duration (Sandelin et al. 2007; Shaw and Valentine 2007; Bystrom et al. 2010). Sequence hydrophobicity also plays a large role in modulating

the aggregation propensity of a protein (Chiti et al. 2003). Several studies have suggested that SOD1 mutations promote exposure of hydrophobic regions that can promote aggregation (Tiwari et al. 2009; Munch and Bertolotti 2010). Taken together, these studies indicate that aggregation is at least partially controlled by the physicochemical properties of amino acid residues within a polypeptide sequence (Chiti et al. 2003; Tartaglia et al. 2008).
In addition, solution conditions can modulate the stability, conformation, and the intermolecular interactions of a protein in solution, and can thereby influence the rate of protein aggregation and the type of aggregate structure formed (Chi et al. 2003; Mahler et al. 2009). Importantly, variations in solution conditions can cause the same protein to aggregate by fundamentally different mechanisms (Goers et al. 2002; Vetri and Militello 2005; Necula et al. 2007). Temperature, pH, macromolecular crowding, agitation, and ionic strength are all variables that can influence aggregation (Chi et al. 2003; Munishkina et al. 2004; Mahler et al. 2009; Sicorello et al. 2009). A number of studies have used different solution conditions (increased temperature, decreased pH, increased ionic strength, sonication or agitation) to promote the formation of well-structured, fibrillar amyloid aggregates (see 1.2.3) by various forms of SOD1 (Stathopulos et al. 2004; Chattopadhyay et al. 2008; Chattopadhyay and Valentine 2009; Oztug Durer et al. 2009). Other studies have demonstrated soluble oligomer and small aggregate formation by various forms of SOD1 in quiescent, physiologically relevant solution conditions (Vassall, 2011, Hwang, 2010, Banci, 2008). Thus, it is evident that multiple factors can greatly influence protein folding and aggregation and these factors must be considered when investigating the molecular mechanisms of protein aggregation.

1.2.3 Amyloid formation

Protein aggregation is a general term that describes a number of diverse processes that culminate in the formation of non-native, multimeric complexes of varied conformations. These aggregates can range from small, soluble oligomers, to larger amorphous structures, and insoluble, well-structured fibrils (Uversky and Dunker 2010). Amyloid is a common, well characterized, type of aggregate formed by proteins associated with many diseases, including the neurodegenerative prion, Parkinson's, Alzheimer's, and Huntington's diseases (Chiti and Dobson 2006; Chiti and Dobson 2009). Extensive studies of amyloid have resulted in significant advances in understanding the underlying molecular basis of protein aggregation (Sipe and Cohen 2000; Chiti and Dobson 2006; Eisenberg et al. 2006; Chiti and Dobson 2009). Classically defined amyloid is characterized by an unbranched, fibrillar aggregate morphology, which exhibits green-gold birefringence upon binding Congo red (Sipe and Cohen 2000), a dye used in disease diagnosis, and a cross-β x-ray diffraction pattern due to the presence of β-strands oriented perpendicular to the long axis of the fibre (Serpell 2000). These large aggregates can be extremely stable and unaffected by cellular clearance machinery (Dobson 1999; Knowles et al. 2007). There is extensive evidence that most and perhaps all proteins can form amyloid under suitable, typically destabilizing, conditions (Dobson 1999; Munishkina et al. 2004; Stathopulos et al. 2004). Amyloid formation can arise from association of unstructured, partially folded, or native-like species, and can be prevented by factors that favour native folding (Chiti and Dobson 2009). These include such factors as: interactions with molecular chaperones that can stabilize partially folded conformations and increase the folding rate; and post-translational modifications or ligand binding that can stabilize the native state and prevent unfolding (Dobson 2004; Chiti and Dobson 2009). Protein size is also a factor that modulates the propensity of a protein to

form amyloid fibrils, as it is less energetically favourable for large proteins to form an amyloid core, compared to smaller proteins (Baldwin et al. 2011; Ramshini et al. 2011).
It should be noted that ALS is not classified by pathologists as an amyloid disease (Kerman et al. 2010). Recent studies have reported the formation of SOD1 aggregates *in vitro* that exhibit some features of amyloid (Banci et al. 2008; Furukawa et al. 2008; Oztug Durer et al. 2009); however, the relevance of such studies to human disease is not known. Typically, there is considerable structural heterogeneity in amyloid (Platt and Radford 2009) and in other amorphous or ordered aggregate structures formed by many peptides and proteins (Fink 1998; Seshadri et al. 2009) (see 1.2.4). Careful analyses using multiple probes are required to distinguish between these different aggregate structures. Appropriately characterizing mixtures of aggregate structures is a major, ongoing challenge in the study of protein aggregation.

1.2.4 Protein aggregation heterogeneity and disease complexity

Neurodegenerative disorders characterized by protein misfolding and aggregation, including ALS, commonly display phenotypic diversity, such as variation in the age of onset, the rate of neuronal dysregulation, and the area of the nervous system affected (Armstrong et al. 2000; Goedert et al. 2001; Frost and Diamond 2009; Williamson et al. 2009). Although the molecular origins of such phenotypic diversity are complex and may differ between diseases, in recent years it has been shown that protein aggregates, including amyloid fibrils, exhibit extensive structural heterogeneity both *in vivo* and *in vitro* (Berryman et al. 2009; Frost and Diamond 2009). Not only do fibrils formed by different amino acid sequences adopt conformations that differ in length and twist, but the structure of fibrils formed by the *same* sequence can vary depending on solution conditions (Berryman et al. 2009). Fibres can vary in the number of amino acids that participate in forming the amyloid core, the arrangement of β-strands in a parallel or antiparallel conformation within each protofilament, and the alignment of β-sheets along the protofilament axis (Tycko 2006). The structure that a particular protein adopts prior to aggregation influences the structure of the aggregate formed and the conformational plasticity of a native protein may play a large role in determining the number of structurally different aggregates produced (Jones and Surewicz 2005; Natalello et al. 2008). Although aggregate structures formed from the same protein can be quite diverse (ie. amorphous versus amyloid structures), in many cases the formation of such structures is energetically favourable and therefore switching between aggregate conformations can require a large amount of energy. As a result, a particular fibril can become trapped in a single conformation (Berryman et al. 2009).
Structural heterogeneity of protein aggregates has been known for many years for amyloid fibrils derived from prion proteins, infectious protein agents that give rise to a number of neurodegenerative disorders known as spongiform encephalopathies or prionopathies. In these diseases, the infectious agent is a misfolded prion protein (PrP^{Sc}, S referring to Scrapie, the disease caused by this infectious agent), which once introduced into a host cell can bind to the native prion protein (PrP^{c}, c referring to cellular) and induce conversion to the PrP^{Sc} form, inevitably resulting in the spread of the disease phenotype (Tuite and Serio 2010). Because a prion protein can adopt a number of conformations, there is considerable heterogeneity in the structure of the amyloid fibrils that are formed from these proteins. Prion amyloid fibrils can differ in stability, surface charge and degree of polypeptide incorporation into the amyloid core, differences that may play a large role in determining

rate of prion replication and the strength of the disease phenotype (Verges et al. 2011). In recent years, evidence for conformational diversity, or different strains, of protein aggregates has also been described for non-infectious protein conformational disorders such as Alzheimer's, Parkinson's, Frontal Temporal Dementia, and ALS (Frost and Diamond 2009; Furukawa et al. 2010). The proteins linked to many of these diseases are natively disordered, and so can easily sample different conformations, which may facilitate aggregation via multiple pathways. Mutations and/or post-translational modifications can greatly influence the population of different conformations of a protein, and thus can largely influence the aggregation process. This point may be particularly relevant to ALS toxicity, since SOD1, although natively folded, undergoes extensive post-translational modification *in vivo* (Valentine et al. 2005), and is increasingly malleable in its less stable, immature forms (refer to section 3). How different fALS-associated mutations modulate the stability of different forms of SOD1 may largely determine the ALS phenotype (Vassall et al. 2011).

2. Structure and function of Cu, Zn-superoxide dismutase (SOD1)

Before discussing SOD1 folding, misfolding and aggregation, we will give a brief description of the tertiary and quaternary structure of SOD1. Human SOD1 is a 32 kDa homodimeric metalloenzyme, with each subunit consisting of a 153 amino acid chain that is often N-terminally acetylated, contains a highly conserved, intrasubunit disulfide bond, and binds one Cu ion and one Zn ion (Figure 2A, C).

Each monomer folds into a Greek key β-barrel, comprised of two, four-stranded antiparallel β-sheets arranged at an angle with respect to one another. The β-barrel has a non-continuous topology such that strands 1 through 3 together with strand 6 form the first β-sheet, while strands 4, 5, 7 and 8 form the second β-sheet. The β-strands are connected by seven loops that differ greatly in length. Loop IV and loop VII are known as the metal binding and electrostatic loops, respectively, and play important roles in both stability and catalytic function by binding the metals and forming the catalytic site pocket. In addition to forming the Zn-binding site, Loop IV contains residues that are important for dimer interface and intrasubunit disulfide bond formation, which tethers Loop IV to β-strand 8 (Tainer et al. 1982). Thus, Zn binding, disulfide bond formation and dimerization together stabilize the native conformation of this long loop, greatly affecting the overall stability of the protein. Loop VII mainly plays a functional role, containing charged residues that shield the active site. These charged residues are important for guiding the superoxide anion from the surface of the protein into the active site where the redox active Cu ion is bound (Valentine et al. 2005).

SOD1 is abundant and ubiquitously expressed in the cytosol of aerobic organisms (Brown et al. 2004). Maturation of the protein involves a series of post-translational modifications, which are understood to varying extents. When it is initially synthesized in the reducing environment of the cytosol the protein is thought to adopt a marginally stable, folded, monomer structure with a reduced disulfide bond and no bound metals. How the protein acquires Zn is not known; however, Cu can be acquired by interaction with the copper chaperone for SOD1 (CCS) or by a CCS-independent mechanism that may involve glutathione, but that is not well understood (Leitch et al. 2009). CCS also catalyzes intrasubunit disulfide bond formation in the reducing cellular environment (Leitch et al. 2009). Although the most abundant form of SOD1 is usually the native, fully mature,

dimeric protein (Valentine et al. 2005), there is also evidence for a significant pool of SOD1 that lacks bound Cu, and is activated in response to oxidative stress (Brown et al. 2004). From here we will refer to the various states of the protein in terms of disulfide and metallation status, with a focus on the disulfide reduced (2SH), disulfide oxidized (S-S), fully metallated (holo) and metal free (apo) states.

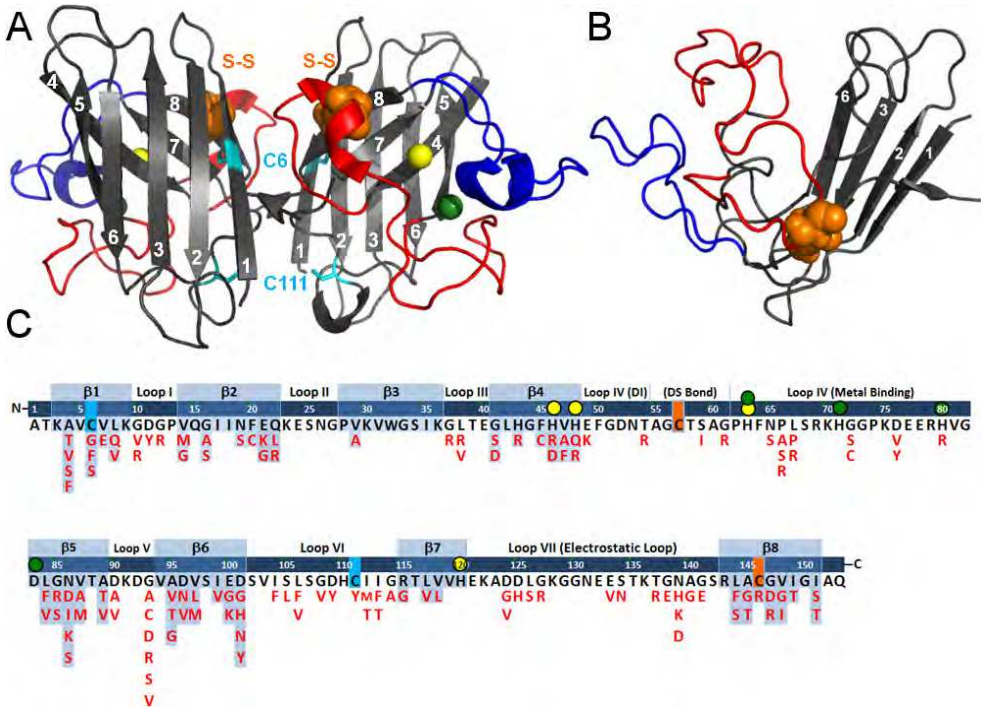

Fig. 2. The structural elements of SOD1.
The crystal structure of fully mature SOD1 is shown in panel A (pdb: 1HL5) (Strange et al. 2003). The β-strands are shown in gray and labelled in white starting from the N-terminus; the functional loops IV and VII are shown in red and blue, respectively; the conserved disulfide bond (S-S) between Cys57 and Cys146 is indicated by orange spheres, the non-conserved free Cys (6 and 111) are shown in cyan; and Cu and Zn are indicated by yellow and green spheres, respectively. The solution structure of the metal free SOD1 monomer variant (pWT*mon*, refer to the introduction of section 3) is shown in panel B (pdb: 1RK7) (Banci et al. 2003). In this structure, only β-strands 1-3 and 6 are well defined. The colour scheme in panel B is identical to panel A. The primary sequence of the SOD1 monomer is shown in panel C. The colours used to depict the secondary structure elements are identical to those used in panels A and B. The positions of known fALS-associated mutations are listed vertically in red below the naturally occurring amino acid in black. The yellow and green spheres indicate the metal binding residues and the secondary structure elements are listed above the primary sequence.

3. Folding, unfolding and misfolding of SOD1

A common and powerful approach to understanding the molecular basis for aggregate formation is to investigate the biophysical properties of mutant proteins in the native and unfolded states, as well as any equilibrium or kinetic intermediates that arise as the protein folds or unfolds (Dobson 2004). Equilibrium species refer to the most stable conformations that are significantly populated under specific steady-state conditions, while kinetic species refer to conformations that are transiently populated as an unfolded protein folds into its native conformation. Typically, kinetic folding intermediates have a relatively low energy barrier of formation, and therefore can form quickly, but they are generally not the most stable conformations. Before protein folding has reached equilibrium (ie. during kinetic conditions), it is not the stability of each state that determines the relative population of each species along the folding/unfolding pathway, but rather how rapidly these states can be accessed on the time scale of protein folding/unfolding. Investigating the molecular characteristics that govern the stability of different states and enable efficient folding of SOD1 can provide key insights into the cause of ALS (Rumfeldt et al. 2006; Stathopulos et al. 2006; Vassall et al. 2006; Rumfeldt et al. 2009; Vassall et al. 2011).

In recent years, systematic analyses of the effects of fALS-associated mutations on the stability and folding of various forms of SOD1, including holoS-S, apoS-S and apoSH, have been reported. Human SOD1 contains two free cysteine residues at amino acid positions 6 and 111 (Figure 2), and these free cysteine residues inhibit reversible unfolding of SOD1 *in vitro* by forming intramolecular and intermolecular non-native disulfide bonds, which promote SOD1 aggregation (Lepock et al. 1990; McRee et al. 1990). Reversible unfolding is a prerequisite for thermodynamic analysis, and so to overcome this limitation pseudo-wild type (pWT) constructs lacking these free cysteines have been used extensively for *in vitro* studies of SOD1. In the most widely used pWT construct, the free cysteines are mutated to alanine and serine at positions 6 and 111, respectively (Lepock et al. 1990; McRee et al. 1990; Stathopulos et al. 2003; Rumfeldt et al. 2006; Stathopulos et al. 2006; Vassall et al. 2006; Kayatekin et al. 2008; Rumfeldt et al. 2009; Vassall et al. 2011); however, other mutations at these positions have also been used (most notably C6A and C111A) (Lindberg et al. 2004; Nordlund and Oliveberg 2006; Nordlund et al. 2009). Not only are these chemically and structurally conservative mutations, a serine at position 111 is found in most other mammalian SOD1, and alanine at position 6 is observed in other non-mammalian organisms (Getzoff et al. 1989). Mutating the free cysteines results in highly reversible unfolding of pWT, while having very minimal effects on structure, function and stability (Lepock et al. 1990; McRee et al. 1990; Hallewell et al. 1991; Parge et al. 1992; Vassall et al. 2011). In addition, an engineered monomer construct (pWT*mon* SOD1) has been used to investigate the effects of ALS mutations on the stability and folding behaviour of individual SOD1 subunits (Nordlund and Oliveberg 2006; Hornberg et al. 2007; Kayatekin et al. 2008; Nordlund et al. 2009; Kayatekin et al. 2010). The monomer construct contains two glutamic acid residues in place of Phe50 and Gly51, and the presence of these charged residues in the dimer interface prevents SOD1 dimerization (Bertini et al. 1994; Banci et al. 1998). The use of both pWT and pWT*mon* SOD1 constructs has provided valuable insights into the mechanism of SOD1 folding and misfolding, which are described in the following sections, starting with the most immature to most mature form of SOD1.

SOD1 Form	t_m pWT (°C)	t_m WT (°C)	ΔG, 37°C pWT (kcal mol^{-1})
HoloS-S	**92.0[a] (81.7[b])**	**80.1[b]**	**33.0[a,c]**
ApoS-S	**59.0[a]**	**52.5[d]**	**13.2[a,c]**
Apo2SH	**47.6[e]**	**46.8[e]**	**1.8[e,f]**

Table 1. Stability parameters obtained from DSC measurements of different forms of wild type (WT) and pWT SOD1
[a](Stathopulos et al. 2006) The parameters were obtained using average fitted values determined using 0.5 mg mL^{-1} protein in 20 mM Hepes pH 7.8.
[b](Lepock et al. 1990) t_m defined as the temperature of half completion of the DSC profiles determined using 2-4 mg mL^{-1} protein in 100 mM phosphate.
[c]ΔG extrapolated to 37°C using methods described in (Stathopulos et al. 2006). Value is in units of per mol dimer.
[d](rodriguiz/valentine,2005,PNAS)determined using 2 mg mL^{-1} protein in 100 mM potassium phosphate pH 7.4.
[e](Vassal,2011,PNAS) parameters obtained using average fitted values determined using 0.5 mg mL^{-1} protein in 20 mM Hepes pH 7.4, 1mM TCEP.
[f]Value is in units of per mol monomer.

3.1 Equilibrium denaturation of apo2SH SOD1

In its most immature form, with no bound metals and reduced disulfide bond, apo2SH SOD1 adopts a marginally stable folded monomer structure. Chemical and thermal equilibrium denaturation of apo2SH SOD1 is well described by a 2-state unfolding transition between folded and unfolded monomers, denoted as M and U, respectively (M ↔ U) (Kayatekin et al. 2010; Vassall et al. 2011). At 37˚C and neutral pH, this form of the protein has a low free energy of unfolding, 1.8 kcal mol^{-1} and 1.6 kcal mol^{-1} for pWT and WT, respectively (Vassall et al. 2011), indicating that ~95 % and 93 % of the protein is folded. Furthermore, the corresponding melting temperatures are 48 °C and 47 °C, respectively. This stability is relatively low compared to other globular proteins, which typically have unfolding free energies of ~5-15 kcal mol^{-1} (Jackson 1998) as well as to more mature forms of SOD1 (see sections 3.2-3.4, 3.6, 3.7, Table 1).
Structural investigations by x-ray crystallography and NMR have shown that without the bound metals and disulfide bond, the interface loop (Loop IV) is minimally structured and the dimer interface is disrupted (Arnesano et al. 2004; Hornberg et al. 2007). The NMR solution structure of monomeric (pWT*mon*) with no bound metals but intact disulfide bond, apoS-S (Banci et al. 2003) provides an interesting comparison (Figure 2B). pWT*mon* apoS-S SOD1 adopts an open β-barrel structure due to the flexibility of β-strands 4 and 5, and the inability of the two β-sheets to effectively pack against one another. Furthermore, Loops IV and VII are extensively disordered (Banci et al. 2003; Banci et al. 2010). Disulfide bond reduction promotes further disorder of the marginally stable Loop IV structure by releasing it from its anchor to β-strand 8 (Hornberg et al. 2007). Because Loop IV contains residues required for Zn binding, disulfide bond formation and dimerization, these modifications are thermodynamically coupled. When the disulfide bond is reduced, SOD1 has a much lower affinity for Zn (75 nM *verses* 100 pM for apoS-S SOD1) (Kayatekin et al. 2010), and dimer formation is energetically unfavourable (Arnesano et al. 2004).

Diverse fALS-associated mutations have markedly different effects on the stability and the folding reversibility of apo2SH SOD1 (Kayatekin et al. 2010; Vassall et al. 2011). Wild-type, pWT, and pWT*mon* apo2SH SOD1 are predominantly folded and can undergo reversible chemical and thermal denaturation, and hence resist aggregation from partially or fully unfolded states (Kayatekin et al. 2010; Vassall et al. 2011). Various fALS-associated mutations decrease the unfolding reversibility, which precludes determination of the free energy of unfolding, and suggests that these mutations increase the aggregation propensity of apo2SH SOD1 (Vassall et al. 2011). Interestingly, an increased tendency to aggregate is not observed for all fALS-associated mutants, in particular not for those with compromised metal binding (Kayatekin et al. 2010; Vassall et al. 2011). Although the free energy of unfolding could not be determined for all mutants studied, owing to the limited stability of this form of the protein, the effects of each mutation on stability could be estimated from their apparent melting temperatures. This revealed that fALS-associated mutations generally have the largest effect on the stability of this most immature form of SOD1 (Table 1), often decreasing the melting temperature of apoSH to below 37°C, and so markedly increasing the fraction of protein that is unfolded at physiological temperature (Furukawa and O'Halloran 2005; Kayatekin et al. 2010; Vassall et al. 2011). Of the mutant SOD1s that unfold reversibly, most show decreased thermodynamic stability, but some (e.g. those involved in metal binding) have little or even a stabilizing effect on stability (Valentine et al. 2005; Kayatekin et al. 2010; Vassall et al. 2011). These findings imply that in some cases the key effects of mutations in ALS may be manifested in more mature forms of SOD1.

3.2 Kinetic unfolding and refolding of apo2SH

Relatively little is known about the kinetic folding mechanism of apo2SH SOD1. Initial studies of engineered reduced monomer variants have reported 2-state (U ↔ M) folding kinetics, which resembles the behaviour of monomeric apoS-S SOD1s (Lindberg et al. 2004; Kayatekin et al. 2010) (see section 3.4). A study using a monomeric C6A/C111A/C57A/C146A construct that resembles the apo2SH form, due to its inability to form a disulfide bond and dimerize, showed that disulfide bond formation was not required to facilitate the early contacts made in the monomer folding pathway (Lindberg et al. 2004). Thus, the transition state between unfolded and folded monomers in both the apo2SH and the apoS-S monomer folding pathways may be similar. However, the disulfide bond stabilizes the folded monomer by decreasing the rate of unfolding, thereby increasing the population of folded monomer (Lindberg et al. 2004) (see section 3.4).

3.3 Equilibrium denaturation of apoS-S SOD1

Formation of a disulfide bond between Cys57 and Cys146 greatly diminishes the conformational freedom of Loop IV (Hornberg et al. 2007), and gives rise to energetically favourable dimer formation (Lindberg et al. 2004; Vassall et al. 2006; Ding and Dokholyan 2008; Kayatekin et al. 2010; Vassall et al. 2011). The observed equilibrium folding mechanism of the pWT apoS-S SOD1 dimer depends on the method of inducing denaturation (i.e. chemical denaturant *versus* heat). Spectroscopically-monitored chemical denaturation of pWT apoS-S SOD1 can be described by a 3-state mechanism in which dimer dissociation is followed by monomer unfolding ($N_2 \leftrightarrow 2M \leftrightarrow 2U$) (Vassall et al. 2006). Due to mass action, however, at increased protein concentrations there is little population of the folded monomer and the mechanism appears 2-state ($N_2 \leftrightarrow 2U$) (Lindberg et al. 2004; Svensson et

al. 2006; Vassall et al. 2006). Similarly, due to the higher stability of the apoS-S dimer compared to the apoS-S monomer and the high protein concentration requirement, thermal denaturation by differential scanning calorimetry (DSC) of apoS-S pWT SOD1 appears 2-state ($N_2 \leftrightarrow 2U$) and so does not provide direct information about the energetics of dimer dissocation ($N_2 \leftrightarrow 2M$) (Stathopulos et al. 2006; Vassall et al. 2006). Thermal denaturation does reveal, however, that the melting temperatures of wild-type and pWT apoS-S SOD1 are approximately 53°C (Rodriguez et al. 2005) and 60°C (Stathopulos et al. 2006), respectively; therefore, the oxidized form of the protein is predominantly folded at physiological temperature. The differences in the reported melting temperatures may be related to different buffer conditions used as well as the folding irreversibility of wild-type SOD1 (Lepock et al. 1992; Chrunyk and Wetzel 1993; Stathopulos et al. 2003). The changes in melting temperatures caused by fALS mutations generally range from -15°C to +2°C; thus, apoS-S SOD1 mutants are also mainly folded at physiological temperature (Rodriguez et al. 2005; Vassall et al. 2006; Kayatekin et al. 2010).

The stability as well as the conformational dynamics of a protein can be assessed using hydrogen-deuterium (H/D) exchange measurements, which can identify regions of the protein undergoing structural opening (Bai et al. 1995). Interestingly, a number of fALS-associated mutants, in particular those that compromise metal binding, display a similar exchange rate as wild type (Rodriguez et al. 2005). However, others increase the rate of structural fluctuations of apoS-S SOD1 (Rodriguez et al. 2005; Prudencio et al. 2009). In particular, some mutants show pronounced opening of the β-barrel around the edge strands at physiological temperatures (Prudencio et al. 2009). Thus, both equilibrium denaturation and H/D exchange experiments reveal that fALS-associated mutations have diverse effects on the stability of apoS-S SOD1, ranging from destabilizing to stabilizing.

Highly reversible chemical denaturation behaviour has enabled accurate measurements of the energetics of both dimer dissociation and monomer unfolding for pWT and mutant apoS-S. Determining how mutations affect the energy, and thus the population, of each species formed along the (un)folding pathway, provides insight into the mechanisms of apoS-S aggregation. Chemical denaturation experiments have revealed that for structurally and chemically diverse mutations, fALS-associated mutations generally decrease the stability of apoS-S SOD1 by destabilizing both the monomer and the dimer interface, with a larger affect on monomer stability compared to the dimer stability (Vassall et al. 2006). Remarkably, the effects of the mutations appear to propagate extensively through the apoS-S form of the protein, inevitably destabilizing the dimer interface (Vassall, K.A. et al. unpublished data) (Khare et al. 2006; Bystrom et al. 2010). Structurally, the apoS-S SOD1 dimer interface is small compared to the amount of solvent exposed surface area (Tainer et al. 1982; Parge et al. 1992); consequently, the dimer interface may be more easily perturbed in the apoS-S state. Moreover, metal loss induces asymmetry in the dynamics of the apoS-S SOD1 monomers, indicative of a structure that is less compact than the holoS-S dimer (Strange et al. 2007). The conformation of both the β-barrel core, in particular β-strand 5, and the functional Loops IV and VII are less rigid in the absence of bound metal (Banci et al. 2009; Teilum et al. 2009). It has been proposed that metal binding is important for shielding the charged residues in Loops IV and VII, which contain too few hydrophobic residues to facilitate close packing with the β-barrel core in the absence of bound metal (Nordlund et al. 2009). Similarly, metal binding may protect against conformational changes in SOD1 that increase hydrophobic exposure (Tiwari et al. 2009).

Therefore, it seems reasonable that many fALS-associated mutations have a large effect on the global stability of apoS-S SOD1. Structural perturbations in one region of the apoS-S SOD1 structure are likely to result in structural perturbations in another region. However, it is clear that fALS-associated mutations have differing effects on the global stability of apoS-S SOD1. In particular, both the apoS-S and apo2SH forms of the metal-binding mutants appear to possess similar stability compared to the wild-type protein (Rodriguez et al. 2005; Vassall et al. 2011). In fact, some fALS-associated mutations increase the stability of apo2SH and apoS-S forms compared to pWT SOD1 (Vassall et al. unpublished data) (Vassall et al. 2011). For example, the mutation H46R increases the stability of both apo2SH and apoS-S SOD1 and the reason for this increase in stability may be due to the introduction of a positively charged side chain into the metal binding pocket of the protein, effectively mimicking the stabilizing effects of the charged metals.

3.4 Kinetic unfolding and refolding of apoS-S SOD1

The kinetic unfolding mechanism of pWT apoS-S SOD1 can be described by the same 3-state mechanism previously outlined for equilibrium denaturation of the wild-type and pWT apoS-S form, where dimer dissociation is followed by the unfolding of two monomers (Figure 3) (Svensson et al. 2006). The overall observed rate of unfolding depends on four microscopic rate constants: the rate constant for folding (k_f), unfolding (k_u), monomer association (k_a), and dimer dissociation (k_d) (Figure 3B); however, under highly denaturing conditions (ie. high denaturant concentration), the marginally stable monomer unfolds rapidly and the rate of the entire unfolding pathway is determined by the rate of dimer dissociation (k_d) (Lindberg et al. 2004; Svensson et al. 2006). In contrast, the overall rate of the refolding pathway of wild-type and pWT apoS-S is limited only by the rate of monomer folding. Therefore, once the monomer has folded the rate of monomer association is extremely rapid (Lindberg et al. 2004; Svensson et al. 2006). The transition state for monomer association is similar to the native dimer with respect to the amount of buried surface area (Svensson et al. 2006). This is similar to the transition state between unfolded and folded monomer, which represents the major energetic barrier of the unfolding/refolding pathway. It has been observed that ~70% of the structure that is buried in the monomer intermediate is also buried in the transition state between the unfolded and folded monomer, suggesting that considerable structural rearrangements must occur before that transition state can form (Svensson et al. 2006). Interestingly, under both equilibrium and kinetic conditions, the population of monomer intermediate remains below 0.5% at physiologically relevant protein concentrations, suggesting that the folding pathway of SOD1 has evolved to limit accumulation of marginally stable monomer intermediates (Svensson et al. 2006). Equilibrium denaturation analyses of fALS-associated mutant apoS-S SOD1 demonstrate a decreased stability of both the monomer intermediate and the dimer interface, increasing the population of monomeric intermediate as well as the unfolded monomer (Lindberg et al. 2004; Vassall et al. 2006), which enhances the accessibility of partially folded, high energy states that may give rise to aggregation.

Kinetic unfolding/refolding studies of the monomeric apoS-S SOD1 (pWT*mon*) and the apo2SH forms of SOD1 can be compared to gain an understanding of how disulfide bond formation modulates the SOD1 folding pathway. Both the pWT apo2SH and the pWT*mon* apoS-S forms of SOD1 fold via a 2-state mechanism, whereby the unfolded monomer adopts a folded conformation in one step (U ↔ M) (Lindberg et al. 2004; Nordlund and Oliveberg

2006; Kayatekin et al. 2010). Owing to the similar folding/unfolding mechanism of apo2SH and pWT*mon* apoS-S, disulfide bond formation is probably not requisite in the early monomer folding events. Consistent with this notion, the topology of the β-barrel brings Cys57 and Cys146 close in space, promoting disulfide bond formation. Although reduction of the disulfide bond does not prevent formation of the necessary contacts that facilitate monomer folding, the maintenance of the disulfide bond modulates the stability of the SOD1 monomer by decreasing the rate of unfolding and to a smaller extent increasing the rate of folding (Lindberg et al. 2004; Kayatekin et al. 2010).

Removal of Loops IV and VII has little effect on the structure and dynamics of the core apoS-S β-barrel (Nordlund et al. 2009). Additionally, the folding behaviour of pWT*mon* apoS-S SOD1 in the absence of these functional loops remains 2-state; however, this SOD1 construct has a 10-fold increase in the rate of folding, while the rate of unfolding is less affected (Nordlund et al. 2009). Removing the Zn-binding site has little effect on the rate of folding, but decreases the rate of unfolding, stabilizing the folded apoS-S SOD1 monomer (Nordlund et al. 2009). These results suggest that while the β-barrel can fold independently of Loop IV and VII, these critical functional loops endow the protein with a less than optimal folding mechanism and may increase the aggregation propensity of the immature forms of the protein.

The overall rate of both apo2SH and apoS-S SOD1 folding appears to be dictated largely by the structural determinants of the monomer folding nucleus. Furthermore, regions extraneous to this folding nucleus are the more labile regions of the protein that unfold first, and thus have been suggested to play a role in SOD1 aggregation. It has been shown that β-strands 1-3 of the first β-sheet must make contact with β-strands 4 and 7 in the second β-sheet to overcome the monomer folding energy barrier. The other strands (β5, β6 and β8) remain disordered in the transition state between unfolded and folded monomers (Nordlund and Oliveberg 2006). By attaching Loop IV to β-strand 8 and forming the dimer interface the disulfide bond may prevent the structural fluctuations that lead to the unravelling of the more dynamic strands of the β-barrel.

3.5 Are the metal free forms of SOD1 the common denominator in fALS toxicity?

Taken together, equilibrium and kinetic folding studies of apo2SH and apoS-S SOD1 suggest that a number of factors may contribute to an increased tendency to aggregate. Many fALS-associated mutations increase the fraction of partially folded monomers, while a few mutations remain wild-type-like in their folding behaviour and aggregation propensity. Because the β-strands display differences in conformational freedom, the location of each fALS-associated mutation and the physicochemical properties of the amino acid introduced seem to greatly affect SOD1 stability and accessibility to partially folded species that may bridge the gap between productive folding and aggregation pathways. These differences may also change the structural properties of the aggregates formed. In recent years, many studies have focused on characterizing a common underlying cause of toxicity in all SOD1-associated fALS cases. Thus a great deal of attention has been directed towards studying the biophysical properties of the more immature forms of SOD1, since fALS-associated mutations have a greater effect on the stability of these forms compared to the fully mature holoS-S form (Lindberg et al. 2004; Furukawa and O'Halloran 2005; Furukawa and O'Halloran 2006; Khare et al. 2006; Nordlund and Oliveberg 2006; Svensson et al. 2006; Smith et al. 2007; Furukawa et al. 2008; Nordlund et al. 2009; Oztug Durer et al. 2009; Tiwari

et al. 2009; Kayatekin et al. 2010; Vassall et al. 2011). Despite this recent focus, the form of SOD1 that is most relevant to ALS pathogenesis remains unknown. It is clear that some fALS-associated mutations minimally affect the stability and folding kinetics of apo2SH and apoS-S SOD1, which suggests that it is necessary to look beyond the immature forms of SOD to uncover the cause of ALS.

3.6 Equilibrium denaturation of holoS-S SOD1

Similar to the equilibrium denaturation pathway of apoS-S SOD1, the observed equilibrium denaturation mechanism of holoS-S SOD1 depends on the mode of denaturation and protein concentration. For pWT SOD1, holoS-S equilibrium denaturation curves fit a reversible 3-state model in which the folded holo native dimer transitions to the unfolded monomer through a folded, metallated, monomeric intermediate (Rumfeldt et al. 2006). The presence of bound metal stabilizes the monomer intermediate far more than the dimer interface. Thus, the dimer is only slightly stronger in holoS-S compared to apoS-S, while the free energy of monomer folding is much higher for holoS-S SOD1 compared to apoS-S SOD1 (Rumfeldt et al. 2006; Vassall et al. 2006). At high protein concentrations, the population of the monomer intermediate is significantly reduced and the equilibrium denaturation mechanism approaches 2-state (Rumfeldt et al. 2006). Similarly, in thermal denaturation by DSC, the monomeric intermediate is not significantly populated and the unfolding appears 2-state (Stathopulos et al. 2006). In both chemical and thermal denaturation of holoS-S SOD1, metals remain bound throughout the transition, although binding is weakened in the monomer intermediate and unfolded state compared to the dimer (Rumfeldt et al. 2006; Stathopulos et al. 2006; Kayatekin et al. 2008). Equilibrium denaturation of holoS-S SOD1 reveals that metal binding significantly increases the free energy of unfolding (Table 1). Consistent with this, the melting temperature of holoS-S SOD1 is ~30°C higher than apoS-S SOD1. Thus, it is likely that the stabilizing effects of metallation as well as disulfide bond formation are intrinsic inhibitors of SOD1 aggregation. Mutations therefore may exert toxicity to the cell by inhibiting SOD1 maturation and/or by promoting metal loss, dimer dissociation and/or disulfide reduction (Tiwari and Hayward 2003; Lindberg et al. 2004; Furukawa and O'Halloran 2005; Furukawa and O'Halloran 2006; Banci et al. 2007; Hornberg et al. 2007; Ding and Dokholyan 2008; Furukawa et al. 2008; Tiwari et al. 2009).

Comparable to apoS-S SOD1, the equilibrium denaturation mechanism of fALS-associated mutant holoS-S SOD1 remains the same as pWT. In a number of cases mutations have been shown to decrease the stability of holoS-S, by decreasing the stability of the monomer, with less effect on dimerization (Rumfeldt et al. 2006; Stathopulos et al. 2006; Vassall et al. 2006). This decreased stability of the holoS-S monomer can often be attributed to weakened metal binding (Hayward et al. 2002), as metal dissociation results in an increased population of the less stable apo state. Thus, in most cases mutations appear to have a more local effect on the stability of holoS-S SOD1 compared to apoS-S SOD1 (Rumfeldt et al. 2006; Vassall et al. 2006); yet, structural perturbations due to mutation may propagate further if metal binding is compromised since loop dynamics and interface stability are greatly affected by the presence of metals (Valentine et al. 2005; Smith et al. 2007; Museth et al. 2009). The overwhelming majority of fALS-associated mutations destabilize the holo state, but because of its extremely high thermodynamic stability the absolute increase in the amount of unfolded species will still be very small, and thus unlikely to affect aggregation. What seems more likely to impact disease is increased local structural fluctuations that can arise from

metal loss and/or dimer dissociation, exposing regions of the SOD1 structure that can make favourable contacts with other SOD1 molecules, and thereby give rise to aggregation from native-like states (Elam et al. 2003; Hwang et al. 2010).

3.7 Kinetic unfolding and refolding of holoS-S SOD1

Analyzing the kinetics of holoS-S SOD1 folding and unfolding provides a method for further characterizing the marginally stable intermediates that form along the folding pathway as these are not always detected at equilibrium where only the most stable species are measurably populated. These kinetic intermediates nevertheless may play significant roles in holoS-S SOD1 aggregation. Both pWT and wild-type holoS-S kinetically unfold slowly *in vitro* via a monomeric intermediate species which has somewhat weaker metal binding affinity relative to the native dimer (Rumfeldt et al. 2006; Kayatekin et al. 2008; Mulligan et al. 2008; Rumfeldt et al. 2009). The overall rate of holoS-S unfolding is dependent on the microscopic rate constants that define each equilibrium transition (Figure 3A).

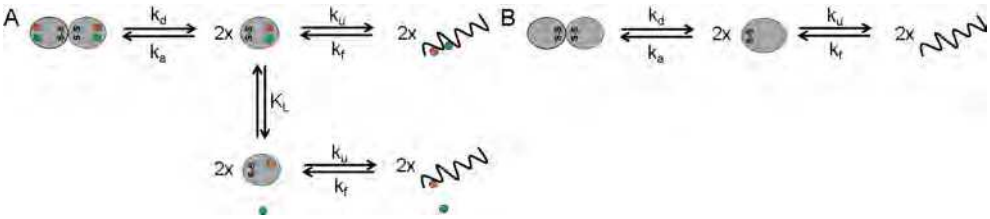

Fig. 3. The kinetic unfolding mechanism of holoS-S and apoS-S SOD1.
In panel A, the kinetic unfolding mechanism of holoS-S SOD1 is shown. The overall rate of unfolding is dependent on the microscopic rate constants k_d, k_a, k_u, k_f, and protein and metal concentration, as well as the equilibrium constant for Zn dissociation from the monomeric intermediate. For a more detailed description refer to (Rumfeldt et al. 2009). In panel B, the simpler kinetic unfolding mechanism of apoS-S SOD1 is shown. The overall rate of unfolding depends only on the microscopic rate constants k_d, k_a, k_u, k_f and protein concentration.

The observed unfolding kinetics therefore depend on the rate of dimer dissociation, which occurs rapidly, and the rate of monomer unfolding, a much slower process (Lynch et al. 2004; Rumfeldt et al. 2009). Under high protein concentration conditions, or in the presence of excess metals, pWT holoS-S SOD1 remains fully metallated as it unfolds, with metals remaining bound to the unfolded state, while at low protein concentrations, Zn can dissociate from both the monomeric intermediate and unfolded monomer (Rumfeldt et al. 2009). Similar results were obtained in kinetic unfolding studies of wild-type holoS-S SOD1 in the presence of a metal chelator, where dimer dissociation and Zn loss were found to occur simultaneously, followed by a conformational change in the β-barrel that precedes and facilitates Cu loss (Mulligan et al. 2008).
The differences in Cu and Zn binding as holoS-S SOD1 unfolds can be rationalized by considering the structural differences in the Cu and Zn binding sites, as follows. While SOD1 binds both metals with extremely high affinity, Cu binds with higher affinity than Zn,

with dissociation constants (K_d) estimated as 10^{-18} M and 10^{-14} M (Crow et al. 1997), respectively. In principal, structural differences between the transition state resembling the free energy barrier between the folded and unfolded monomer, compared to the structure of the folded monomer, determine the regions of the protein that, if changed, will have the largest effect on the rate of unfolding. If a particular region is structured in both the monomer and the transition state, then alterations in the stability of that region, either by metal binding or mutation, will affect the free energy of the monomer and its unfolding transition state in a similar way. As a result, the difference in free energy between the folded monomer and transition state and therefore the rate of monomer unfolding will be unaffected. Alternatively, if a particular region of the protein is structured in the folded monomer, but not in the transition state, then changes in the stability of that region will affect the energetics of the folded monomer and transition state differently. In this case the rate of unfolding will be affected. The Cu binding site is formed by residues in β-strands 4 and 7, which are thought to be structured in the transition state, while the Zn binding site is formed mainly by residues in Loop IV and β-strand 5, which are thought to be disordered in the transition state (Nordlund and Oliveberg 2006). Therefore, the rate of unfolding should be affected more by Zn binding than Cu binding and indeed there is some experimental evidence that suggests this to be the case (Rumfeldt et al. 2009).

It has been shown that fALS-associated mutant holoS-S SOD1s increase the unfolding rates of holoS-S SOD1 (Rumfeldt et al. 2009; Ip et al. 2010). Increased unfolding rates and accessibility of either on or off-folding pathway intermediates may increase the accessibility of transient protein species that can initiate aggregation (Dobson 2003; Wang et al. 2008). Furthermore, it has been proposed that fALS-associated mutations, even those far from the metal binding sites, promote increased levels of Cu-deficient intermediates along the holoS-S SOD1 unfolding pathway (Ip et al. 2010). Cu-deficient intermediates are lower in stability and therefore may have a higher tendency to aggregate.

The *in vitro* refolding mechanism of holoS-S SOD1 is more complex than apoS-S SOD1 due to the presence of metal (Figure 3). While the apoS-S form folds via a simple 3-state (2-step) mechanism, with a rate constant that is limited by the rate of monomer folding (see section 3.4), holoS-S refolding occurs through parallel pathways that differ with respect to the fraction of metal bound to the unfolded and transition states (Rumfeldt et al. 2009). However, in the presence of excess Cu and Zn and at high protein concentrations, conditions that favour metal binding to the unfolded state, the refolding kinetics can be described as a simple 2-step process, as each unfolded SOD1 monomer is saturated with metal. Zn coordination in the native binding site stabilizes the monomer intermediate and folded dimer more than the unfolded monomer, accelerating refolding of apoS-S 100 fold (Kayatekin et al. 2008). However, Zn can also bind to non-native sites on the protein (Kayatekin et al. 2008; Nordlund et al. 2009). The Cu-coordinating residues are capable of binding Zn with micromolar affinity in the denatured state after mutation of the native Zn coordinating residues (Nordlund et al. 2009). This non-native coordination of Zn augments the stability of apoS-S SOD1 by increasing the rate of folding and decreasing the rate of unfolding, but forces SOD1 to adopt a non-native conformation. Thus misligation of Zn can cause misfolding and decrease the efficiency of folding (Nordlund et al. 2009). However, with intact Cu and Zn binding sites, Zn may be coordinated by the Cu-binding site first, helping to overcome the main energy barrier of folding, before moving to the Zn-binding site (Nordlund et al. 2009). Other studies have demonstrated that Zn-binding is required to

pre-organize the Cu-binding site (Banci et al. 2003). Together these studies show that metal binding significantly modulates the efficiency of the SOD1 folding pathway by stabilizing both the monomer and dimer and decreasing the rate of unfolding. However, non-native metal binding may force the protein to be kinetically trapped in a partially folded, aggregation prone conformation that is more stable than the unfolded state (Nordlund et al. 2009). Together these results show that the kinetic unfolding and refolding mechanism of SOD1 is highly dependent on metal binding.

3.8 fALS mutations and modifications have complex effects on the folding and stability of SOD1

It is evident that fALS-associated mutants have different and complex effects on the stability of SOD1, and these effects depend on the form of SOD1 being studied. For example, mutations that have a large effect on holoS-S SOD1 stability, such as metal binding mutants, tend to have a much smaller effect on the more immature forms of the protein (Valentine et al. 2005). Also, the effects of mutations are more pronounced, but to varying extents for different mutations, with decreasing stability in immature forms of SOD1 (Vassall et al. 2006; Vassall et al. 2011). It is important to understand how both the equilibrium and kinetic folding pathways of all forms of SOD1 are altered by fALS-associated mutations to untangle the complexity of SOD1 aggregation. Certain mutations may have a large effect on the thermodynamic stability of the protein, through weakened metal binding or by decreasing the stability of the dimer interface, while having a smaller effect on the kinetic stability of SOD1. Other mutations may only subtly alter the thermodynamic stability of the native state, exerting their effects by altering the kinetic stability of SOD1 by decreasing the rate of folding, or by increasing the rate of unfolding (Rumfeldt et al. 2006). These effects can increase the equilibrium and/or transient population of folded or unfolded monomeric species that are prone to aggregate (see section 4).

In addition, fALS mutations may alter the susceptibility of SOD1 to post-translational modifications which will tend to decrease protein stability. These include not only enhanced metal loss and disulfide reduction (resulting in higher population of immature species), but also other modifications, in particular ones that are enhanced under oxidizing cellular conditions, which may occur late in disease. Examples include cysteine oxidation (Gruzman et al. 2007; Karch et al. 2009; Bosco et al. 2010), glutathionylation (Proctor et al. 2011), tryptophan oxidation (Elam et al. 2003), and glycation (Meiering 2008). While there is relatively little quantitative data on the effects of these modifications on stability, there is evidence that they can be destabilizing. Such modifications may play different roles at different disease stages; for example, oxidative modifications may become more pronounced as the disease progresses and contribute to rapid disease progression by enhancing formation of toxic aggregates (Karch et al. 2009).

4. SOD1 aggregation

Numerous experimental studies have characterized the aggregation of multiple forms of SOD1. These are described below, focussing mainly on *in vitro* studies, and their implications for disease. It is important to note, however, that it is not yet known what forms of SOD1 are involved in human disease: neither the disulfide bond status nor metal content of SOD1 in aggregates is known. Evidence supports the population of multiple

forms of SOD1 *in vivo* (see section 2). Collectively, experimental findings provide support for contributions from many forms of SOD1 to toxic aggregation in ALS.

4.1 Aggregation of Apo2SH SOD1

4.1.1 Evidence of Apo2SH SOD1 aggregation

Aggregation of the marginally stable apoSH form of SOD1 has been suggested in a number of studies to be particularly important in ALS pathogenesis. Studies from multiple groups have reported that wild-type, pWT and fALS-associated mutant apoSH SOD1 are all predisposed to aggregate (Lindberg et al. 2004; Furukawa and O'Halloran 2005; Hornberg et al. 2007; Chattopadhyay et al. 2008; Furukawa et al. 2008; Oztug Durer et al. 2009; Vassall et al. 2011). Additional support for the biological significance of apoSH aggregation is that fALS-associated mutants expressed in insect cells tend to be more metal deficient and disulfide reduced compared to wild type; this finding was proposed to be a consequence of enhanced opening of the mutant SOD1 structures, exposing the disulfide bond to the reducing environment of the cytosol (Tiwari and Hayward 2003). Furthermore, murine models of fALS have revealed the presence of aggregated disulfide reduced SOD1 species (Jonsson et al. 2006; Zetterstrom et al. 2007). Based on what is known about the relationship between protein stability and aggregation propensity (see section 1.2.1), and on the relatively low stability and the expanded, fluctuating structure of apoSH SOD1 (see section 3.1), it appears that aggregation of this form of SOD1 could be significant under cellular conditions (Furukawa et al. 2008). Consistent with this idea, *in vitro* agitation of apo2SH SOD1 results in amyloid fibril formation (Chattopadhyay et al. 2008; Oztug Durer et al. 2009; Furukawa et al. 2010). Although the agitation phenomenon is poorly understood at a molecular level it may favour amyloid fibril formation over pathways to other morphologies. Agitation may enhance aggregation in general by promoting the formation of aggregation-prone species at air-solution or solid-solution interfaces, as well as increase the rate of aggregation by distributing aggregation nuclei more efficiently and causing preformed aggregates to break and create new nucleation sites (Sicorello et al. 2009). Again, it should be noted that since agitation can promote aggregation of many proteins (Rousseau et al. 2008; Mahler et al. 2009; Sicorello et al. 2009), and ALS is not classified as an amyloid disease (Kerman et al. 2010), it is not yet clear how agitation-induced aggregation *in vitro* is related to aggregation in disease.

4.1.2 Mechanisms of Apo2SH SOD1 aggregation

Intriguing structural variations in apoSH amyloid and non-amyloid aggregates have been reported and may be related to the different disease durations for different SOD1 mutants. Structural diversity has been observed in the amyloid fibrils formed by different SOD1 mutants (Furukawa et al. 2010). In addition, *in vitro* aggregation experiments without agitation showed that different apo2SH SOD1 mutants may form different sized, small (~40 nm - 1000 nm), soluble, non-amyloid aggregates (Vassall et al. 2011). These soluble species may be particularly relevant to fALS toxicity, as oligomeric protein aggregates have been implicated as key neurotoxic species in many other neurodegenerative diseases (Caughey and Lansbury 2003; Soto and Estrada 2008). The variations in structural characteristics for apo2SH SOD1s aggregates may indicate that different mutants favour distinct aggregation pathways, depending on the aggregation-prone conformations that mutants may adopt.

Another factor in the heterogeneity of aggregate structures formed may be that apo2SH is appreciably unfolded at physiological temperatures, and therefore may sample multiple aggregation-prone conformations.

4.2 Aggregation of ApoS-S SOD1

4.2.1 Evidence of ApoS-S SOD1 aggregation

Aggregation of the apo S-S form of SOD1 has been studied extensively *in vitro*. This is likely related to the reasonable ease of preparation and aggregation of this quite stable form of the protein (Table 1) (see section 3.3 and 3.4). A role for apoS-S in disease is supported by various *in vitro* and *in vivo* evidence for aggregation of metal-deficient SOD1 as well as evidence that mutations can promote loss of bound metals (Valentine et al. 2005; Molnar et al. 2009; Oztug Durer et al. 2009; Hwang et al. 2010; Lelie et al. 2011). Both wild-type and fALS mutant apoS-S SOD1s are predominantly folded, but mutations can significantly increase the population of folded and unfolded monomeric species (Lindberg et al. 2004; Svensson et al. 2006; Vassall et al. 2006), and increase structural fluctuations (Ding and Dokholyan 2008; Teilum et al. 2009), both of which can promote aggregation. The formation of the disulfide bond decreases the propensity of apoS-S to aggregate compared to apoSH (see section 4.1), while the absence of bound metals in apoS-S SOD1 increases its aggregation propensity compared to holoS-S (see also section 4.3).

Several studies have described the *in vitro* formation of amyloid-like soluble or fibrillar aggregates for wild-type and fALS-associated apoS-S SOD1 mutants (Furukawa and O'Halloran 2005; Banci et al. 2007; Banci et al. 2008; Oztug Durer et al. 2009). Aggregation of mutant SOD1 in these studies was often accelerated by agitation, resulting in the formation of aberrant disulfide bonds between Cys6 and Cys111 (Figure 2) and removal of the free thiol groups by mutation (Banci et al. 2007; Cozzolino et al. 2008) generally diminished aggregation. The role of aberrant disulfide bond formation has been investigated extensively using fALS mutant SOD1 mice models of ALS where it appears that such bonds are observed mainly late in disease (Cozzolino et al. 2008; Karch and Borchelt 2008). These results highlight an important question: does the role of SOD1 differ at different stages of disease? Considerable evidence suggests that the answer is yes; in particular, soluble oligomers may be particularly important early in disease, whereas larger aggregates become prominent after the onset of disease symptoms (Boillee et al. 2006; Cozzolino et al. 2008; Turner and Talbot 2008). Many questions on the roles of different aggregate species and their targets remain unanswered.

A number of studies have reported aggregation of apoS-S SOD1 in the absence of aberrant disulfide bond formation. Formation of such amyloid-like aggregates by pWT and mutant SOD1 can be induced by agitation at neutral pH; however, in these studies the observed ease of aggregation was not correlated with disease duration (Chattopadhyay et al. 2008; Furukawa et al. 2008; Oztug Durer et al. 2009; Furukawa et al. 2010). Also, soluble, reversible aggregates induced by heat were detected by DSC (Vassall et al. unpublished data) (Stathopulos et al. 2006; Vassall et al. 2006). In these studies, mutations in a pWT background generally increased aggregate formation. Furthermore, decreased stability of fALS-associated mutant apoS-S SOD1 was correlated with increased formation of amorphous aggregates that mature into fibril structures that resemble those found in fALS patients (Stathopulos et al. 2003).

4.2.2 Mechanisms of ApoS-S Aggregation

A number of studies have focussed on possible molecular mechanisms of apoS-S SOD1 aggregation, often based on various structural and dynamic data. In the absence of bound metal, there is still extensive disorder in the functional Loops IV and VII, and this disorder may promote exposure of the β-barrel core and deprotection of the β-barrel edge strands (β-strands 5 and 6) (Strange et al. 2007). With these edge strands exposed, H-bonding ligands within the strands are free to interact with the edge strands of other SOD1 molecules and these aberrant contacts can lead to fibril formation (Elam et al. 2003; Nordlund and Oliveberg 2006). As an evolutionary strategy to avoid this fibrillation mechanism, the edge strands of β-proteins often contain charged "gatekeeper" residues, advantageously positioned to disfavour β-sheet extension (Otzen et al. 2000). Protection of the β-barrel edge strands in SOD1 is facilitated both by charged residues in β-strands 5 and 6 and by Loops IV and VII, which block the edge of the β-barrel in the holoS-S form (Nordlund and Oliveberg 2006). The other edge of the β-barrel is buried within the dimer interface. Therefore, the dynamics of Loop IV and VII that cause exposure of either β-strands 5 and 6, or the dimer interface, may lead to fibrillation. Solution NMR experiments of wild-type apoS-S SOD1 suggest that disorder in Loops IV and VII allows stretches of amino acids within these functional loops to form additional β-strands, thereby initiating oligomerization (Banci et al. 2010).

In addition, β-strands 4 and 5 are connected by Loop IV and β-strands 7 and 8 by Loop VII, and the disorder in these long loops appears to propagate to the flanking β-strands. Consequently, the β-sheet formed by strands 4, 5, 7 and 8 is less defined in apoS-S SOD1 compared to the β-sheet formed by strands 1-3 and 6 (Banci et al. 2003; Banci et al. 2010). Thus, metal loss and increased mobility of Loops IV and VII may result in exposure of hydrophobic residues in the β-barrel core (Tiwari et al. 2009). In addition, β-strands 1-3 have been proposed to be a nucleation site for aggregation of apoS-S as they form a continuous patch of hydrophobic residues (Nordlund and Oliveberg 2006). These 3 strands form early in the monomer folding pathway (Lindberg et al. 2004; Nordlund and Oliveberg 2006) and therefore may become exposed by partial unfolding of the apoS-S protein.

The destabilizing effects of fALS-associated mutations appear to propagate significantly in apoS-S SOD1, in particular generally weakening the dimer interface (see section 3.3) (Vassall et al. unpublished data) (Khare et al. 2006; Bystrom et al. 2010). Destabilization of the apoS-S dimer interface can increase the levels of marginally stable, apoS-S monomers. Furthermore, it was demonstrated recently that breathing motions of the wild-type apoS-S monomer result in transient formation of a higher energy species with weakened packing and a partially exposed hydrophobic core (Teilum et al. 2009). fALS-mutations induce further perturbations in this higher energy state that open up the structure of the mutant apoS-S SOD1 monomer more compared to wild type (Teilum et al. 2009) and so may further promote aggregation.

The increased structural dynamics observed for metal-free SOD1 described above, suggests that apoS-S may be predisposed to aggregate compared to the holoS-S state. Several studies have demonstrated that apoS-S SOD1 can aggregate by a variety of different mechanisms that give rise to different aggregate morphologies, including both disulphide cross-linked and non cross-linked species, amyloid-like fibrils and non-amyloid amorphous aggregates.

4.3 Aggregation of SOD1 from the holoS-S state

4.3.1 Evidence of aggregation from the holoS-S state

While the highly stable, native holoS-S form of SOD1 (see section 3.5) generally appears to be much less susceptible to aggregation than other forms of the protein (Stathopulos et al. 2003; Valentine et al. 2005), there is evidence that a number of SOD1 mutants can give rise to aggregation from the holoS-S form. Hwang et al. found that prolonged incubation of both pWT and fALS-associated holoS-S SOD1 mutants at physiological temperature and pH results in changes in metal binding and/or dimerization, diminished specific dismutase activity, and the nucleated formation of low levels of amorphous aggregates (Hwang et al. 2010). Furthermore, these experiments show that, although the aggregated SOD1 demonstrated some metal loss, there was still a significant amount of metal bound, indicating that complete metal loss was not essential for aggregation. Although both pWT and mutant holoS-S SOD1 were observed to aggregate, in general the holoS-S SOD1 mutants lose specific activity quicker, and aggregate more rapidly, and to a greater extent, than pWT. Importantly, the aggregates formed from holoS-S SOD1 in this study exhibited similar structural, dye-binding, and immunological characteristics as the aggregates found in fALS patients (Hwang et al. 2010). In contrast, other studies have reported that SOD1 does not aggregate from the holoS-S form (Chattopadhyay et al. 2008), or requires extremely destabilizing conditions with agitation to promote fibrilization (Oztug Durer et al. 2009). The differences between these findings may be related to the different experimental conditions for studying SOD1 aggregation, such as length of incubation, frequency of sampling, and methods for monitoring aggregation.

4.3.2 Mechanisms of holoS-S aggregation

Immature forms of SOD1 can form amyloid fibrils far more readily than holoS-S (Banci et al. 2007; Furukawa et al. 2008; Oztug Durer et al. 2009), and this difference in aggregation tendency is likely related to the very high stability and rigidity of holoS-S compared to the less mature forms (Stathopulos et al. 2003; Rumfeldt et al. 2006; Stathopulos et al. 2006; Svensson et al. 2006; Vassall et al. 2006; Furukawa et al. 2008; Kayatekin et al. 2008; Kayatekin et al. 2010; Vassall et al. 2011). Highly disordered, predominantly unfolded, proteins tend to favour the formation of amyloid (as may be the case for apo forms of SOD1), whereas more structured proteins favour formation of amorphous aggregates (as for holoS-S) (Munishkina et al. 2004). Measurements of global thermodynamic stability have shown that, owing to the high stability of the holo form, destabilizing mutations will in general cause very small increases in the population of unfolded protein (Rumfeldt et al. 2006; Stathopulos et al. 2006); these increases are unlikely to account for SOD1 aggregation in ALS. Aggregation may alternatively arise from native-like, locally unfolded states (Chiti and Dobson 2009; Hwang et al. 2010) (Figure 1B) which appear to be enhanced in holoS-S SOD1 by some fALS-associated mutations (Shipp et al. 2003; Hough et al. 2004; Banci et al. 2005; Museth et al. 2009). Ultimately, it is likely that some sort of relatively rare/slow structural change is required to bring about aggregation from holoS-S SOD1 (Hwang et al. 2010), in contrast to the apo2SH form, which aggregates readily for some fALS-associated mutant SOD1s (Vassall et al. 2011).

SOD1 aggregation arising from the holoS-S form appears to occur through a nucleation-dependent mechanism that is characterized by a lag phase (i.e. slow nucleation) followed by fast aggregate growth (Hwang et al. 2010). The lag phase corresponds to the time required

for holoS-S SOD1 to arrange into an aggregation-prone state and/or form the necessary contacts required for aggregation. It is likely that dimer dissociation and/or metal loss from SOD1 occur during this lag phase and may be important triggers of aggregation (Hwang et al. 2010). Furthermore, various fALS-associated mutations appear to decrease the length of the lag phase, perhaps due to weakened metal binding and/or a weakened dimer interface, (Crow et al. 1997; Khare et al. 2004; Tiwari et al. 2009).

These results suggest that fully mature SOD1 is not devoid of the ability to aggregate, as it could give rise to native-like aggregation-prone species via loss of metal, dimer dissociation, or local structural openings, promoted by mutation. Such aggregation may be highly relevant to fALS toxicity, since holo S-S is generally the most highly abundant form of SOD1 *in vivo* (Valentine et al. 2005).

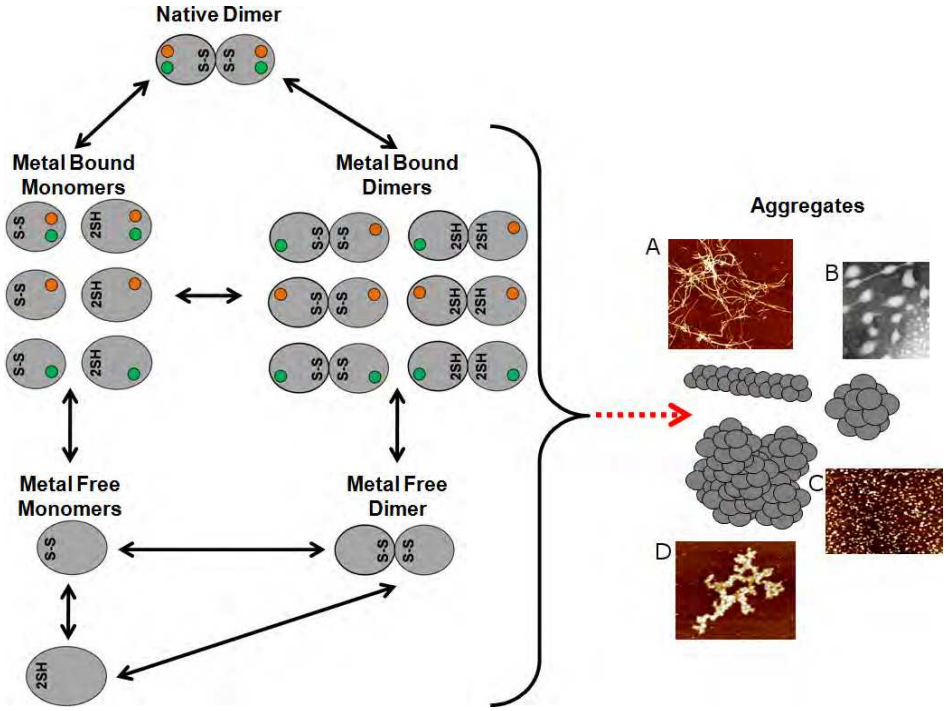

Fig. 4. Many forms of SOD1 may be relevant to ALS toxicity.

SOD1 can exist in many forms *in vivo*, which is illustrated in Figure 4. Each monomer is depicted as a grey sphere that is smaller when metals are bound and/or the disulfide is formed. The presence of Cu and Zn is shown by orange and green spheres, respectively; and S-S and 2SH indicate disulfide oxidized and reduced species, respectively. The difference in SOD1 conformation prior to aggregation may largely influence the morphology of the aggregates formed. Images and schematic representation of possible aggregate morphologies are shown in the centre on the right. Panels A, C and D are Atomic Force Microscopy images of SOD1 aggregates formed *in vitro* (Broom et al, unpublished data) (Hwang et al. 2010) and panel B is an electron microscopy image obtain from of SOD1 aggregates formed *in vitro* (Stathopulos et al. 2003).

5. Conclusion

Numerous studies have revealed that the effects of fALS-associated mutations on the folding, unfolding and aggregation of different forms of SOD1 are highly complex. Mutations can alter both equilibrium stability, in terms of the energetics of dimer dissociation, monomer intermediate stability, and metal binding, and kinetic stability, in terms of the rates of interconversion between various SOD1 species (Section 3). As a consequence, the populations of various aggregation prone species may be increased for different mutations, and this may give rise to different aggregate structures.

There have been a number of attempts to identify the relationships between the effects of the mutations and ALS disease characteristics. In particular, disease duration, which is characteristic for patients carrying a given SOD1 mutation, has been used as a measure of the toxicity of each fALS-associated SOD1 mutation. Early work focused on the loss of superoxide dismutase activity, and increased oxidative stress as the common underlying cause of disease (Valentine et al. 2005). Subsequently, the focus shifted to the toxic gain of function for mutant SOD1, both aberrant enzymatic SOD1 activity, or increased SOD1 aggregation, the latter being the predominant focus of this review. Owing to the high stability and lower aggregation propensity of the holoS-S form, many studies have focused on characterizing the stability and aggregation mechanisms of the more immature, metal deficient SOD1 forms. However, recent work suggests that disease duration does not correlate strongly with the stability of the apoS-S form of mutant SOD1 (Bystrom et al. 2010). This observation was rationalized by considering the role of factors beyond destabilization in modulating aggregation, such as changes in protein net charge and hydrogen bonding. An interesting study by Wang et al. reported that predicted aggregation propensity, based on the physicochemical properties of the polypeptide sequence (Chiti et al. 2003) combined with the stability of mutant apoS-S SOD1 in a summative score and weighted towards mutants with more patient data, correlated fairly well with fALS disease durations (Wang et al. 2008). On the other hand, recent work by Vassall et al. demonstrated that observed aggregation of the apo2SH form is not correlated with disease duration (Vassall et al. 2011). Collectively, these studies demonstrate that multiple factors including protein stability, dynamics, and biophysical characteristics are likely to play a role in modulating SOD1 aggregation, and that fALS phenotypic characteristics are not likely to be fully explained by the aggregation behaviour of any one form of SOD1.

Aggregation studies on holoS-S, apoS-S, and apo2SH SOD1 mutants have identified multiple mechanisms and aggregate morphologies (Section 4 and Figure 4). HoloS-S SOD1, widely thought believed to be much less susceptible to aggregation, has nevertheless been shown to form amorphous aggregates in a nucleation-dependent manner where the lag phase may involve metal loss or monomerization (Hwang et al. 2010). ApoS-S SOD1 may form amyloid- or non-amyloid-like aggregates with or without disulphide cross-linking depending on the solution conditions, and apo2SH SOD1 has been found to adopt the most diverse range of aggregate morphologies, including soluble aggregates under physiologically relevant conditions which may be particularly neurotoxic (Caughey and Lansbury 2003). Considering the influence of SOD1 mutations on the stability, unfolding and folding patterns of all forms of SOD, together with the diverse mechanisms of aggregation, different mutations may be influencing the protein in variable ways, resulting in a wide spectrum of effects. This diversity is likely to play a significant role in the variable disease courses for fALS patients with SOD1 mutations. Ultimately, the role of SOD1 in ALS

may be similar to the roles of other globular, oligomeric proteins in misfolding diseases such as: transthyretin in familial amyloidotic polyneuropathy and senile systemic amyloidosis, lysozyme in hereditary non-neuropathic systemic amyloidosis, immunoglobulin light chain in monoclonal protein systemic amyloidosis, prion protein in Kreutzfeld Jakob, and serpins in serpinopathies (Ohnishi and Takano 2004; Harrison et al. 2007). In these diseases mutations are generally destabilizing, but the extent of destabilization of monomer versus subunit interfaces varies widely. The role of SOD1 in disease may be further complicated by the potential aberrant enzymatic activity of misfolded and/or aggregated species which could cause oxidative damage. In addition, it is worth considering the different roles of various types of SOD1 aggregate structures, or contributions of aberrant activity and the effects of these on other cellular components, at different stages throughout the disease course of ALS. For these reasons, it is important that future studies continue to consider the possible roles of multiple forms of SOD1 mutants in modulating the formation of different aggregate structures (Figure 4). A combination of further *in vitro* and *in vivo* studies of folding and aggregation will be critical for untangling the role of toxic aggregation in the syndrome of ALS.

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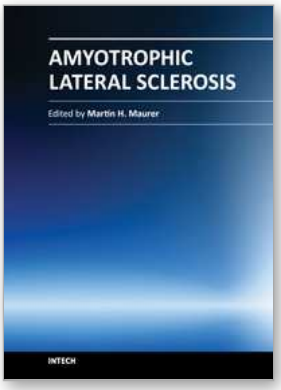

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Though considerable amount of research, both pre-clinical and clinical, has been conducted during recent years, Amyotrophic Lateral Sclerosis (ALS) remains one of the mysterious diseases of the 21st century. Great efforts have been made to develop pathophysiological models and to clarify the underlying pathology, and with novel instruments in genetics and transgenic techniques, the aim for finding a durable cure comes into scope. On the other hand, most pharmacological trials failed to show a benefit for ALS patients. In this book, the reader will find a compilation of state-of-the-art reviews about the etiology, epidemiology, and pathophysiology of ALS, the molecular basis of disease progression and clinical manifestations, the genetics familial ALS, as well as novel diagnostic criteria in the field of electrophysiology. An overview over all relevant pharmacological trials in ALS patients is also included, while the book concludes with a discussion on current advances and future trends in ALS research.

How to reference

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