2008

Characterization of human copper/zinc superoxide dismutase

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DOI: <https://doi.org/10.31979/etd.r7ms-5pe6>
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- Yamazaki, K.; Iwura, T.; Ishikawa, R.; Ozaki, Y. Methanol-induced tertiary and secondary structure changes of granulocyte-colony stimulating factor. *J. Biochem. (Tokyo)* **2006**, 140, 49-56.
- Zhang, F.; Zhu, H. Intracellular conformational alterations of mutant SOD1 and the implications for ALS-associated SOD1 mutant induced motor neuron cell death. *Biochim. Biophys. Acta*, **2006**, 1760, 404-414.
- Zhou, H. X.; Dill, K. A. Stabilization of proteins in confined spaces. *Biochemistry* **2001**, 40, 11289-11293.
- Zimmerman, S. B.; Minton, A. P. Macromolecular crowding: biochemical, biophysical and physiological consequences. *Annu. Rev. Biophys. Biomol. Struct.* **1993**, 22, 27-65.

CHARACTERIZATION OF HUMAN COPPER/ZINC SUPEROXIDE DISMUTASE

A Thesis

Presented to

The Faculty of the Department of Chemistry

San José State University

In Partial Fulfillment

of the Requirements for the Degree

Masters of Science

by

Nada Khouderchah

December 2008

UMI Number: 1463390

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ABSTRACT

CHARACTERIZATION OF HUMAN COPPER/ZINC SUPEROXIDE DISMUTASE

by Nada Khouderchah

In this research, both human wild type (WT) and mutant A4V SOD1 were encapsulated in wet-aged sol-gel glasses in an attempt to identify intermediates leading to aggregation. The encapsulated proteins were subjected to various conditions known to cause aggregation in solution such as heat, low pH ~ 4.0 , EDTA, TCEP or DTT, and TFE. Changes in the secondary structure of the entrapped SOD1 were monitored by using circular dichroism (CD) in the far-UV region.

Results showed major differences between the WT and A4V SOD1 secondary structures under most conditions. The encapsulation method is shown to enable long-term monitoring of the secondary structural changes over several months, and the optimal encapsulation method for the SOD1 is identified in order to minimize leaching. Finally, a possible common intermediate for both A4V and WT SOD1 was identified under different conditions.

ACKNOWLEDGMENTS

I would like to express my gratitude to my advisor, Dr. Daryl Eggers, whose expertise, guidance, and understanding added considerably to my graduate experience. I appreciate his assistance in editing this thesis. Also, I would like to thank the other members of my committee, Dr. Brooke Lustig and Dr. Gilles Muller, for their valuable input and advice.

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CHAPTER 1

1.0 Introduction

1.1 Overview

Amyotrophic lateral sclerosis (ALS), also called Lou Gehrig's disease, is a progressive neurodegenerative disease that targets, and eventually destroys, the motor neurons in the spinal cord and brainstem (Bruijn et al., 2004). People of all races and ethnic backgrounds are affected. ALS cases are generally classified into two different groups: familial ALS (FALS), where a family history of the disease can be traced to a genetic defect, and sporadic ALS (SALS) (Rosen et al., 1993). FALS represents about 5%-10% of the total number of ALS cases. In 20%-25% of FALS cases, the cause of the disease has been found to be associated with a genetic defect on chromosome 21 that codes for an important antioxidant enzyme called copper-zinc superoxide dismutase (CuZnSOD) (Bruijn et al., 2004; Valentine et al., 2005; Andersen et al., 2006). Even though this percentage is very small (1%-2.5% of the total number of ALS cases), understanding the mechanism by which the mutant SOD1 causes the disease may be a crucial step to unlocking the mystery of ALS in general. In fact, the common clinical symptoms of FALS and SALS suggest that the disease mechanism could also be common (Li et al., 1988). Thus, the effects of SOD1 mutations at the cellular and biological level may be relevant not only in FALS cases but in other ALS cases as well.

The discovery of mutations in SOD1 that provided the first insight into the molecular basis of ALS was reported in 1993 (Rosen et al., 1993). To date, over 100 different mutations have been identified and linked to ALS (<http://www.alsod.org>). Unfortunately, these mutations are widely scattered throughout the SOD1 structure,

which does not help in isolating a common denominator of the disease mechanism. Nevertheless, most of the evidence suggests that these mutations cause the neurodegenerative disease by adding a toxic function to the protein (Valentine et al., 2005). This toxic property leads to an increased propensity for the pathogenic SOD1 to aggregate relative to the wild type (WT) protein (Ray et al., 2004; Meiering 2008) and to form amyloid-like fibrils (Bruijn et al., 2004). Aggregation usually occurs when a protein misfolds to a non-native conformation and fails to be degraded or to refold back to the native conformation. Consequently, the protein aggregate becomes the direct or indirect cause of the pathological conditions related to the disease (Dobson, 1999; Wang et al., 2008). Indeed, it was reported that the SOD1 mutants adopted different conformations than WT *in vivo*. These altered conformations of SOD1 mutants were observed prior to the formation of protein aggregates (Zhang and Zhu, 2006). Also, the crystal structures of two different mutants, A4V and I113T, showed large conformational changes as compared with the WT SOD1 in solutions (Hough et al., 2004). These results suggest that the conformational changes and the aggregate formation play an important role in the origin of the disease. Therefore, the detection and isolation of intermediates that could lead to aggregation are the obvious next steps. Our current knowledge of the pathogenic mechanisms of ALS is based mostly on studies with various mutant SOD1s in solution where the protein tends to aggregate with itself rather quickly (Valentine et al., 2005; Banci et al., 2007; Cao et al., 2008). Thus, an alternate approach is needed to facilitate the detection of the intermediates leading to aggregation.

A summary of the latest literature related to this thesis is provided in this chapter, including the functions, structures, and findings of the protein SOD1 (WT and A4V mutant), as well as the sol-gel method of protein encapsulation.

1.2 Overview of Experimental Goals

The objective of this research is to detect possible intermediate(s) that could lead to protein aggregation by using the sol-gel encapsulation method. In this study, we encapsulate human SOD1 WT and mutant A4V proteins in silica glasses where every protein molecule is isolated in an attempt to prevent protein aggregation while applying various conditions that normally lead to aggregation in solution. The conditions used were: lowering the pH to 3.5, using the metal chelator ethylenediaminetetraacetic acid (EDTA) (DiDonato et al., 2003), adding mild denaturants such as 2, 2, 2-trifluoroethanol (TFE), using heat treatment (Stathopoulos et al., 2003), or reducing the disulfide bond with either dithiothreitol (DTT) or Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Tiwari and Hayward 2003; Arnesano et al., 2004). Also, the effect of the salt

tetramethylammonium chloride $N(CH_3)_4Cl$ on SOD1 was investigated. Circular dichroism (CD) was used to observe conformational changes in the secondary structure of the encapsulated proteins.

1.3 Structure of the Protein

The human enzyme CuZnSOD is a 32-kDa homodimer with ellipsoidal dimensions of about 30x40x70 Å in size. SOD1 is a stable enzyme. It is resistant to proteolysis and to inactivation by chemical denaturants such as 4% sodium dodecyl

sulfate (SDS) and 10 M urea (Forman and Fridovich, 1973; Marklund et al., 1997). In addition, fully metallated SOD1 unfolds at a temperature above 80°C (Rodriguez et al., 2002). The unusual stability of SOD1 is due to its structural properties, which consist of an eight-stranded β -barrel motif, binding sites for copper and zinc ions, an intrasubunit disulfide bond, hydrophobic interactions, and a network of hydrogen bonds (Valentine et al., 2005; Shaw and Valentine 2006).

SOD1 is a dimeric protein. Each subunit contains 153 amino acid residues, an internal disulfide bond, one copper and one zinc ion (Parge et al., 1992) (see Figure 1).

Figure 1. Crystal structure of metal bound human SOD1 (figure from PDB, ID 2C9V). Copper ions are shown as orange spheres, and zinc ions as blue spheres.

The two subunits are held together by hydrophobic interactions (Lynch et al., 2004). Each subunit is arranged in a Greek key β -barrel structure composed of eight

antiparallel β -strands joined by seven external loops. Loops I, II and V form connections between adjacent strands. Loop IV (residues 49-84) is involved in interactions between the two subunits and contains Cys⁵⁷, the zinc binding domain and part of the active site cavity. Cys⁵⁷ forms an internal disulfide bond with Cys¹⁴⁶. Loop VII (residues 121-144) is the electrostatic loop that plays a role in substrate attraction. Loop VI (residues 102-114) contributes to the stability of the enzyme. Specifically, Leu¹⁰⁶ and Leu³⁸ act to stabilize the opposite ends of the β -barrel fold and the dimer interface. Each amino acid points its side-chain into the core of the β -barrel and stabilizes the structure through hydrophobic interactions (DiDonato et al., 2003; Fujiwara et al., 2005). Loop VI together with loop III (residues 37-40) forms the Greek-key connection across the β -barrel (Parge et al., 1992) (see Figure 2).

Figure 2. Schematic drawing of the SOD1 structure, as adapted from Parge et al., 1992. β -sheets are shown as open arrows and enumerated in chronological order using Arabic numbers. Loops are drawn as lines and numbered using Roman numerals.

The H-bond network contributes to the protein stability. For instance, a network of hydrogen bonds stabilizes the β -barrel structure and forms bridges between the loops, metal binding sites and structural elements of the subunit. Asp¹²⁴ in the electrostatic loop

is hydrogen bonded to the non-ligating nitrogens of the copper ligand His⁴⁶ and the zinc ligand His⁷¹. Also, the carbonyl oxygen on Gly¹⁴¹ in the electrostatic loop is hydrogen bonded to the non-ligating nitrogen of copper ligand His¹²⁰. Furthermore, the copper binding site is connected to both the electrostatic and the disulfide loops through hydrogen bonds between the non-ligating nitrogen of His⁴⁸, a copper ligand, and the carbonyl oxygen of Gly⁶¹, which is hydrogen bonded with the catalytically important Arg¹⁴³. In turn, Arg¹⁴³ is hydrogen bonded to the carbonyl oxygen of Cys⁵⁷ positioned in the disulfide loop and compromises part of the dimer interface. Also, Cys⁵⁷ is involved in the intrasubunit disulfide bond with Cys¹⁴⁶ located in β -sheet 8. Moreover, part of β -sheet 8 is hydrogen bonded to Gly¹¹⁴ and Arg¹¹⁵ in the Greek key loop VI (Parge et al., 1992; Valentine et al., 2005). So as observed, the hydrogen bond network connects and stabilizes the monomer subunit of hSOD1. Furthermore, the binding of metals as well as the formation of intrasubunit disulfide bond between Cys⁵⁷ and Cys¹⁴⁶ stabilize the dimer structure and shift the equilibrium from monomer to dimer species.

1.3.1 Role of Metals

Each subunit contains one copper and one zinc ion. While the copper is coordinated by four histidine ligands (His⁴⁶, His⁴⁸, His⁶³ and His¹²⁰) and one water molecule, the zinc ion is coordinated by three histidine ligands (His⁶⁰, His⁷¹ and His⁸⁰) and one Asp⁸³. The copper and zinc ions share an imidazolate ligand, His⁶³ (Parge et al., 1992) (see Figure 3).

The copper ion is solvent exposed, whereas the zinc ion is buried inside the β -barrel (Fridovich, 1975). The copper ion is responsible for the catalytic activity of the hSOD1 protein. The Zn^{2+} ion plays a role in stabilizing the structure, adding a positive charge (Banci et al., 2003) and providing, in yeast and human, the correct structure for productive interaction with its copper chaperone (CCS) (Furukawa et al., 2004). This suggests that the zinc ion is inserted before the copper ion. At this time, the process of zinc binding and the source of zinc ion *in vivo* are still unknown (Banci et al., 2003). Once the copper ion is added, the disulfide bond formation between Cys⁵⁷ and Cys¹⁴⁶ follows, and dimerization occurs in the holo-SOD1 protein (holo refers to fully-metallated SOD1 protein).

Figure 3. Schematic drawing of the metal binding of hSOD1 in the oxidized form. The Cu^{2+} is coordinated to one water molecule, three histidines 46, 48 and 120, and shares an imidazolate ring histidine 63 with the Zn^{2+} . In addition, the Zn^{2+} is coordinated to one aspartic acid and two histidines, 71 and 80.

In the absence of metals, SOD1 is in the immature, apoprotein state. The disulfide-reduced apo-form of SOD1 is considered to be the best substrate to enter into the mitochondria, but it is the form most prone to aggregation (Furukawa et al., 2004). In the apoprotein, loop IV, which is involved in the dimerization, and loop VII, the electrostatic loop, become highly disordered and flexible. Under these conditions the two β -sheets look like an “open clam”, and the buried zinc site becomes solvent exposed. Subsequently, Zn^{2+} can be added to the immature, reduced form of the enzyme, restoring order to the two loops (Banci et al., 2003) and inducing the dimeric state (Arnesano et al., 2004). Recent findings have shown that upon the addition of just one zinc ion to the homodimer, the two loops become ordered, and possible communication between the two subunits occurs through contact between the zinc loop and the dimer interface (Potter et al., 2007). However, if the “open clam” configuration should occur in the monomeric species, then the edge β -strands 5 and 6 of one monomer could interact with the edge strands of another monomer, leading to nonproductive protein-protein interactions (Valentine and Hart, 2003) such as those observed in fibril formation in ALS patients (Stathopoulos et al., 2003). It has been shown that the loss of metal ions in mutant SOD1s significantly decreases the thermodynamic stability of the enzyme and leads to a partial unfolding transition and possibly misfolding and aggregation of the apo-SOD1 (Stathopoulos et al., 2003; Lynch et al., 2004; Banci et al., 2008). In the case of A4V apo-SOD1, hydrogen exchange monitored by mass spectroscopy showed that the mutant was destabilized at the dimer contact at residues 50-53 and throughout residues 21-53, which consist of β 3, β 4, loop II and III (Rodriguez et al., 2005; Shaw et al., 2006). These

findings were in line with the various reports indicating that A4V apo-SOD1 aggregated by perturbing the dimer interface (Khare et al., 2004; Rakhit et al., 2004; Ray et al., 2004). Furthermore, differential scanning calorimetry (DSC) studies showed that A4V apo-SOD1 was considerable less stable than WT apo-SOD1 (Rodriguez et al., 2005).

1.3.2 Role of the Cysteine Residues

There are four cysteine residues in the hSOD1 proteins at positions 6, 57, 111 and 146. Cys⁵⁷ and Cys¹⁴⁶ form an intracellular disulfide bond (Tiwari and Hayward, 2003). This bond is a rare feature for an intracellular protein located in a highly reducing environment such as the cytosol (Hwang et al., 1992). When the disulfide bond is formed, it causes a shift in the monomer-dimer equilibrium favoring the dimeric species. Also, it is kinetically stable and, together with the metal ions, contributes to the stability of the enzyme (Arnesano et al., 2004; Furukawa et al., 2004). Hence, when the bond between Cys⁵⁷ and Cys¹⁴⁶ is reduced, loop IV becomes disordered and destabilizes part of the β -barrel, the dimer interface and the zinc-binding site. This could lead to loss of dimerization as well as metal ions, followed by partial unfolding. Indeed, mutant proteins are more susceptible to disulfide reduction and monomerization than the wild-type protein (Arnesano et al., 2004). Recently, it was suggested that Cys⁵⁷ and Cys¹⁴⁶ might play a role in protein aggregation by forming intermolecular disulfide bonds (Deng et al., 2006).

As for the other two-cysteine residues, Cys⁶ is located inside the β -barrel where it is solvent inaccessible and Cys¹¹¹ is solvent exposed and positioned near the dimer

interface (Tiwari and Hayward, 2003). From aggregation studies, it was observed that these two cysteine residues most likely form bonds within the fibrous aggregate and increase the severity of aggregation (DiDonato et al., 2003; Banci et al., 2007) (Figure 4).

Figure 4. Crystal structure of human SOD1 (figure from PDB, ID 1SPD). The cysteines are represented in yellow, and the coordinated metals are shown in green for copper and gray for zinc.

1.4 Function of the SOD1 Protein

Cu/Zn SOD is an antioxidant enzyme found mainly in the cytosol, nucleus, and mitochondrial intermembrane space of eukaryotic cells and in the periplasmic space of bacteria (Valentine et al., 2005). This enzyme has been shown to play a protective role in cells against oxidative stress by catalyzing the disproportionation of superoxide anion into O_2 and H_2O_2 . This reaction occurs through the alternate reduction and oxidation of the active site copper ion. This catalysis is a two-step process (Fridovich, 1995):

In the oxidized form, Cu^{2+} has irregular five-coordinate geometry, and in the reduced form its configuration assumes nearly trigonal planar three-coordination geometry. From the crystal structure of the reduced form, it was shown that Cu^+ moves 1.3 Å away from His⁶³, which becomes protonated, and releases the water ligand. Zn^{2+} has a tetrahedral geometry (Valentine et al., 2005).

To reach the copper ion, the superoxide anion is guided electrostatically through a positively charged, narrow channel (Cudd and Fridovich, 1982). The active-site channel with its bound metal ions is formed between the electrostatic loop VII and loop IV (Parge et al., 1992). Both steps of the reaction occur with a diffusion-controlled rate constant of $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, and each reaction step is pH-independent over the range of 5- 9.5 (Klung-Roth et al., 1973).

1.5 Amyotrophic Lateral Sclerosis (ALS)- the Disease

FALS-linked mutations have been found in every major structural element in the dimeric enzyme such as the β -barrel, loop regions, disulfide bond, copper and zinc binding sites and the dimer interface region (Cleveland and Rothstein, 2001). The majority of these mutations are point mutations in the DNA that cause amino acid substitutions, deletions, or insertions that lead to frameshifts and truncations (Valentine et al., 2005). ALS mutant SOD1 proteins fall into two categories depending on their metal content, SOD activity and spectroscopic characteristics. The first category is known as “wild type-like” (WTL) mutants for which the activity and spectroscopic characteristics of the mutant SOD1 are similar to those of wild-type (WT) SOD1. The second category is comprised of the “metal-binding-region” (MBR) mutants. MBR mutants contain lower

levels of metallation and exhibit different characteristics than the WT SOD1. These mutations are usually found in the metal binding ligands or in the electrostatic and zinc loop regions that are involved in metal binding (Valentine and Hart, 2003). Table 1 lists the 2 categories of FALS mutant SOD1 proteins (Valentine et al., 2005). Now, the puzzling question is how could all these mutations, regardless of type or location, cause the same disease in ALS patients?

Table 1. Lists of FALS mutant SOD1 proteins as adapted from Valentine et al., 2005. The WTL mutants have biophysical characteristics that are close to WT SOD1 protein. The MBR mutants have different biophysical properties than WT SOD1 protein.

Wild-type-like (WTL) mutants				Metal-binding region (MBR) mutants
A4V	G72S	G93V	N139K	H46R
V7E	D76Y	E100G	L144F	H48Q
L8Q	L84V	E100K	L144S	H80R
G37R	N86S	D101N	A145T	G85R
L38V	D90A	D101G	V148G	D124V
G41D	G93A	I113T	I149T	D125H
G41S	G93R	R115G		S134N
H43R	G93C	E133Del		C146R

1.5.1 Gain of a Toxic Function

At this point, it is generally accepted that SOD1 mutations result in a gain, rather than a loss, of some toxic function. These findings have provided support for this hypothesis. First, transgenic mice that expressed both the human FALS mutant G93A

SOD1 and their normal mouse WT SOD1 gene did develop progressive neurodegeneration despite having higher SOD activity. On the other hand, transgenic mice that expressed human WT SOD1 plus their normal mouse WT SOD1 gene did not exhibit ALS symptoms (Gurney et al., 1994), and the SOD1 knock-out mice did not develop motor neuron disease (Reaume et al., 1996). Also, in some FALS mutant SOD1, the enzymatic activity was not affected (Borchelt et al., 1994).

Four major hypotheses have been proposed to explain the toxic gain of function: 1) excitotoxic death due to mishandling of glutamate (Cleveland et al., 2000), 2) neurofilament disorganization leading to axonal strangulation (Hirano et al., 1984), 3) oligomerization (Kaytor and Warren, 1999; Watanabe et al., 2001; Julien, 2001), and 4) oxidative damage (Wiedau-Pazos et al., 1996; Roe et al., 2002). The last two hypotheses have been the main focus of studies of ALS mutant proteins (Rakhit et al., 2002; Valentine and Hart, 2003; Banci et al., 2008; Meiering 2008). The oligomerization hypothesis states that ALS mutant SOD1 proteins become misfolded, oligomerize into high molecular weight species, aggregate with other essential proteins and eventually become proteinaceous inclusions. It is possible that the intracellular aggregate proteins, at some point in their formation, become toxic to motor neurons and cause damage to cellular components and pathways (Valentine and Hart, 2003). Also, it is suggested that the accumulation of aggregated proteins cause oxidative damage. The oxidative damage hypothesis proposes that the misfolding of a mutant SOD1 protein modifies the enzymatic mechanism and allows the protein to catalyze reactions with hydrogen peroxide or peroxynitrite. In turn, reactive species of nitrogen and oxygen become toxic

by inflicting damage to proteins by alteration of amino acid side chains, fragmentation of polypeptide chains, and generation of covalent and noncovalent aggregation (Johnston et al., 2000). The oxidative damage hypothesis requires copper or some other redox active metal ion to be involved in the enzymatic activity. However, some evidence has challenged the validity of the oxidative hypothesis and suggests that the effect of SOD1 mutations in FALS is a result of altered copper chemistry or SOD1 activity (Subramaniam et al., 2002). On the other hand, there is some evidence of damage in the tissues of ALS transgenic mice, caused by elevated oxidative stress (Valentine, 2002). For example, in the G93A mouse, it was found that the mutant CuZnSOD1 protein itself was heavily oxidized (Andrus et al., 1998). Also, oxidative stress may be linked to the accumulation of the unfolded or partially unfolded mutant SOD1s due to decreased proteasome activity and /or degradation of oxidatively damaged SOD1. This could overwhelm or impair the cellular chaperone capacity, cause mitochondria dysfunction, and disrupt other cellular processes (Tiwari and Hayward, 2003). Thus, the oxidative damage theory cannot be discarded. Nevertheless, in each hypothesis misfolding and aggregation are linked to toxicity, and the two hypotheses are most likely intertwined (Valentine and Hart, 2003; Rakhit et al., 2004; Banci et al., 2008) (see Figure. 5).

Figure 5. Diagram showing the possible steps of oligomerization and their connection to the oxidative damage hypothesis.

1.5.2 Aggregation Studies

The toxicity of mutant SOD1 could very well be the outcome of aggregation. Mounting evidence suggests that the aggregation propensity of mutant SOD1 may be the common denominator for all the different mutations (Lindberg et al., 2002; Khare et al., 2004; Rakhit et al., 2004). Stathopoulos and co-workers (2003) showed by using thermal studies of various mutant SOD1s that mutations decreased the protein stability and

increased the unfolding rate. This destabilization increased the propensity of mutants to aggregate (Wang et al., 2008). As indicated in aggregation studies performed in vitro, this process is a multistep reaction that consisted of dimer dissociation, apo-monomer formation followed by aggregation of the monomer species (Khare et al., 2004; Rakhit et al., 2004; Ray et al., 2004). This process is in equilibrium (Khare et al., 2004):

$$\text{Dholo} \leftrightarrow 2\text{Mholo} \leftrightarrow 2\text{Mapo} \rightarrow \text{A}$$
 (Dholo = holo-dimer, Mholo = holo-monomer, Mapo = apo-monomer and A = aggregate). Evidence suggests that the dimer is not the reactive species in aggregation. Instead, the apo-monomer can be a possible intermediate in aggregation. Next, high molecular weight, insoluble protein complexes (IPCs) appear as an early event (Johnston et al., 2000). Then late in the disease, aggresomes or inclusion bodies are formed (Johnston et al., 2000). Inclusion bodies were found in the cytoplasm of motor neurons in ALS patients and transgenic mice expressing human FALS linked SOD1 transgene. SOD1 protein was detected in these inclusion bodies, (Johnston et al., 2000) along with ubiquitin and neurofilament proteins (Valentine and Hart, 2003). The inclusion bodies were associated with amyloid deposits, which were fibrils formed by β -pleated sheets of the protein (Elam et al., 2003).

1.5.3 Mutant Ala 4 Val (A4V)

The A4V mutant is the most common FALS mutation in North America (Stathopoulos et al., 2003). It is associated with the most rapid progression of the disease. The apo-A4V protein has a significantly decreased thermal stability compared to WT SOD1 (Rodriguez et al., 2002, 2005), and exhibits an increased susceptibility to disulfide

reduction (Tiwari and Hayward, 2003). At low protein concentrations, A4V favors the monomer species and aggregates spontaneously in solution at 37°C (Ray et al., 2004). This mutation disrupts the dimer interface, which is formed by reciprocal contacts between the C-terminal β -strand 8 and residues from the disulfide loop (loop IV). The main dimer contacts are residues 50-53, 114, 148 and 150-153. Most of the dimer interface interactions in WT SOD1 are similar in the A4V structure, except at the mutation site and around the N-terminus. It is found that the structure of the metal sites is unaffected by the mutation (Hough et al., 2004), but significant structural changes occur from the increase in volume due to the two additional methyl groups in valine. As a consequence, some bond lengths change, while others are lost or added. Studies revealed that side chains of residues Phe²⁰, Leu¹⁰⁶, Ile¹¹³, Ile¹⁴⁹ and Ile¹⁵¹, which are near Val⁴, shift in a concerted fashion due to steric constraints of the extra two methyl groups on valine and cause changes in the hydrophobic interactions (Cardoso et al., 2002; Hough et al., 2004). In WT SOD1, Ile¹¹³ has a close, single hydrophobic interaction with Ala⁴ with a bond length of 3.6 Å. However in the A4V mutation, Ile¹¹³ moves away from Val⁴, loses the close interaction, and makes an additional bond. This change causes Ile¹⁴⁹ to shift closer to Val⁴, and in turn Ile¹⁵¹ rearranges its position (Hough et al., 2004). Moreover, the subtle change of Phe²⁰ is associated with the rearrangement of the important Leu¹⁰⁶ that protrudes from the β -barrel (Cardoso et al., 2002). These observations indicate that there are significant changes in the overall molecular conformation at the interface, which lead to a different orientation of the two monomers and to destabilization of the dimer interface. As a consequence of this destabilization, a

chain of steps would occur: 1) the monomer-dimer equilibrium would favor the monomer species, 2) subunits would have an increased tendency to lose metal and /or to unfold, (Cardoso et al., 2002; DiDonato et al., 2003; Hough et al., 2004) and 3) the apo-monomer species would be produced, as implicated in aggregation (Khare et al., 2004). When the dimer interface in A4V was stabilized with an engineered intrasubunit disulfide bond, monomerization and aggregation did not occur. Actually, the new A4V became as stable as WT (Ray et al., 2004).

In conclusion, the observations presented here about the mutant A4V follow the general trend that could be a common theme for all or most FALS mutants SOD1s. Hence, using this mutant in this research might be a good model for detecting probable intermediate(s) that lead to aggregation.

1.6 Sol-Gel Encapsulation of Proteins

In this research, the sol-gel method is used to entrap the SOD1 in a nanoporous silica matrix. The advantages and disadvantages of this method are numerous, and the following list highlights only the relevant ones for this investigation.

Advantages:

- The sol-gel method may stabilize the encapsulated protein due to the excluded volume of the silica matrix (Eggers and Valentine, 2001a). The effects of the excluded volume on the protein's structure *in vivo* may play an important role since a large fraction of the available volume in a living cell is occupied by macromolecules (Minton, 2001).

- The sol-gel method allows protein contact with solvents for conformational studies while preventing protein aggregation.
- Glass entrapment generally improves the thermal stability of proteins (Eggers and Valentine, 2001b).
- The protein is encapsulated in a pore size small enough to prevent leaching of the biomolecule, but large enough to allow smaller analytes to enter the matrix.
- The final material is optically transparent, which makes spectroscopic measurements possible (Eggers and Valentine, 2001a; Jin and Brennan, 2002).
- The bovine SOD1 molecule is known to retain its structural integrity and biological function in silica glasses (Ellerby et al., 1992).

Disadvantages:

- Possible leaching of the protein molecules out of the pores (Pierre, 2004).
To overcome this problem, surfactants such as poly(ethylene oxide) could be added to control the pore size (Wei et al., 2002). In this work, protein leaching did occur, and that will be discussed in section 4.1.1.
- The protein can be damaged possibly by the electrostatic interaction with the matrix (Pierre, 2004). Cox and co-workers (2003) used poly(ethyleneimine) to provide protection to the enzyme lactate oxidase.

- Due to the interference of the sol-gel materials in the far-UV, the CD spectra will be stopped at 200 nm. Any readings beyond the 200 nm become unreliable due to the high noise to signal ratio.

In general, the sol-gel process involves the transition of a system from a liquid “sol,” mostly colloidal, into a solid phase. The starting materials or precursors used in the preparation of the sol are usually inorganic metal salts or alkoxysilanes that may have a metal center or functional groups (Wright and Sommerdijk, 2001). Tetramethoxysilane (TMOS) is used in this study. A series of chemical reactions occurs that may proceed simultaneously, starting with hydrolysis, which can be acid or base catalyzed. Then the condensation and polycondensation reactions, which are responsible for building the SiO₂ cage surrounding each biological molecule. Next, gelation occurs such that the mixture does not pour out of the container if tipped. In this step, links form between silica sol particles to form a giant spanning cluster. After that, the aging process takes place where further condensation and polycondensation reactions happen, thus strengthening the matrix material. Also, during this phase the products of hydrolysis and condensation may evaporate, *i.e.*, alcohol and water, causing the matrix and pores to shrink. Overall shrinkage of the material could be up to 85% of the initial volume (Jin and Brennan, 2002; Pierre, 2004). A wet gel can be obtained or a xerogel may be formed by evaporation. Figure 6 shows the different stages of sol-gel processing.

Figure 6. Schematic drawing of the sol-gel steps adapted from Jin and Brennan, 2002. The process begins with hydrolysis, then condensation and polycondensation. Next, the biomolecules are added. Subsequently, gelation and aging occur. A wet gel or xerogel can be obtained, depending on the drying conditions.

1.7 Circular Dichroism (CD)

Circular dichroism (CD) spectroscopy is a form of light absorption spectroscopy that measures the difference in absorbance of right and left circularly polarized light by a molecule which exhibits absorbance and is structurally asymmetric. Asymmetry occurs when a molecule is chiral (non-superimposable on its mirror image), non-chiral but covalently attached to a chiral molecule such as aromatic amino acid side chains, or non-chiral present in an asymmetric environment e.g., a chromophores bound to a protein (Hammes, 2005). CD is well suited to determine and monitor structural and

conformational changes in proteins. The near ultraviolet (UV) region (320-260 nm) provides information about the tertiary structure of the protein, and the far-UV region (260-190 nm) gives information regarding its secondary structure (Sreerama and Woody, 2004). The different secondary structures of proteins are alpha helix, parallel and antiparallel beta sheet, and random coil. Analysis materials have been developed to characterize components of the secondary structures (Sreerama et al., 1999; Sreerama and Woody, 2000). CD data are reported in units of absorbance or ellipticity (θ). Molar ellipticity ($[\theta]$) is normalized for concentration, and is usually expressed in ($\text{deg cm}^2/\text{dmol}$) (Sreerama and Woody, 2004). Before obtaining CD data, pure nitrogen is circulated in the instrument to purge any oxygen from the system, since oxygen absorbs light around 200 nm, and interferes with the measurements.

CHAPTER 2

2.0 Materials and Methods

2.1 Materials

Tris-HCl, methanol, dithiothreitol (DTT), Cu^{2+} sulfate and Zn^{2+} sulfate heptahydrate were purchased from Sigma. Potassium acetate (KOAc) and HCl were bought from Fisher Scientific. Tris (2-carboxyethyl) phosphine hydrochloride (TCEP-HCl) was obtained from Hampton Research. Ethylenediaminetetraacetic acid (EDTA) was purchased from EMScience. Tetramethylorthosilicate 99+ % (TMOS), trimethoxypropylsilane 96% (propylTriMOS), and tetramethylammonium chloride ($\text{N}(\text{CH}_3)_4\text{Cl}$ or TMAcI) were obtained from Aldrich. 2, 2, 2- trifluoroethanol (TFE) was purchased from Fluka. Plastic cassettes of 1.0 mm were bought from Invitrogen. Bovine SOD was purchased from OxisResearch. Human SOD was a generous gift from J. S. Valentine (UCLA) and P. J. Hart (UT Health Science Center, San Antonio). P. J. Hart donated the mutant A4V. Purified water (Milli Q) was used in all the experiments.

2.2 Preparation of Solutions

Tris buffer, 10 mM, at pH 8.0 and 7.0 were prepared. KOAc buffer at 5 mM, pH 5.5, and at 10 mM, pH 5.7, 5.5 and 4.0 were made. Two different disulfide reducing agent solutions were prepared: one was 6 mM DTT at pH 5.5, and the other one 40 mM TCEP at pH 5.5. A salt solution of 1 M $\text{N}(\text{CH}_3)_4\text{Cl}$ at pH 7.0 was prepared. 100 and 1 mM EDTA were used in conjunction with 10 mM KOAc buffer at pH 4.0 for a final concentration of 10 and 0.33 mM EDTA respectively. Protein leaching out of the sol-gel

pores causing loss of the CD signal was a major problem in this work. To circumvent this issue, sol-gel samples were used immediately after the two weeks of maturation, and samples were kept at 4°C at all times (except during incubation periods at 37°C or during testing). As observed in Figure 30, most of the samples had enough protein to give a good CD signal even after 12 days of incubation (except for the sample incubated in 10 mM Tris buffer at pH 8.0).

A series of TFE solutions was prepared from 15-80% (vol/vol) at pH 5.4. A Zn^{2+} sulfate and Cu^{2+} sulfate stock solution of 0.100 M concentration of each was made and then diluted with 5 mM KOAc buffer at pH 5.5 to obtain the desired concentration of 20 mM Zn^{2+} and 20 mM Cu^{2+} at pH 5.5.

Two different disulfide reducing agents, DTT and TCEP-HCl were used because TCEP-HCl has some advantages over DTT. TCEP-HCl is odorless, resistant to air oxidation and is more stable and effective than DTT over a broad range of pH. TCEP-HCl reduces peptides and proteins over a wide range of pH (between 1.5-11.1), salt, detergent and temperature conditions. Also, TCEP-HCl is unreactive toward other functional groups in proteins. Another useful property of TCEP-HCl is the ability to dissolve in water resulting in a solution pH $\sim$ 2.5, which could be adjusted to the desired experimental pH. TCEP-HCl has about 80% of the reducing capacity intact for 21 days over a wide range of pH. TCEP-HCl reduction is rapid when a molar excess is used with the sample. Furthermore, it can be used with IR and NMR spectroscopy (<http://www.hamptonresearch.com>).

2.3 Standard Sol-gel Preparation

Three 15-ml plastic tubes were used per batch. In each tube the following reagents were added in this order: 1.00 mL of TMOS, 30.0 μ L of 0.040 N HCl and 214 μ L of Milli Q water. The acid catalyst HCl was added in this step with the precursor materials to speed up the hydrolysis reactions without denaturing the protein. Next, the solutions were mixed gently, and then placed in ultrasound sonicator filled with ice / water for 20 minutes. Afterward, 4.65 mL of aqueous protein / buffer solution was added to the hydrolysed TMOS mixture of 3.10 mL (determination of protein concentration and aqueous protein / buffer volume are covered in the next section). At this point the entire mixture had a volume of 7.75 mL. Next, the mixture was divided in half and immediately transferred using a plastic pipette into two plastic cassettes. When the mixtures were solidified, the cassettes were covered with parafilm and placed at 4°C for 24 hours. The next day, the methanol-rich liquid that formed on top of the gel was drained, a few milliliters of Milli Q water were added, and the cassettes were covered again with parafilm. The sol-gel mixtures were placed back at 4°C for two weeks to mature (Ellerby et al., 1992; Eggers and Valentine, 2001a) (see Figure 7).

Figure 7. A schematic drawing describing the standard sol-gel preparation.

After two weeks of aging, the sol-gel sheets were ready to be prepared as individual samples for experiments. Each silica sheet was cut vertically about every centimeter with a razor blade to give multiple small wafers that fit into a quartz cuvette with a 2 mm pathlength, as used in CD analysis. Then, each silica wafer was incubated in 3.0 mL of the desired buffer or solution at 4 or 37°C for a minimum of 24 hours to assure the pore volume of the glass has fully equilibrated with the solution (Eggers and Valentine, 2001a). Next, samples were carefully moved to a cuvette using forceps and

immersed in their original buffer or solution (unless otherwise specified), and CD spectra in the far-UV were recorded using the circular dichroism spectrophotometer, AVIV Model 215. Data were collected from 200 to 260 nm at 25°C unless mentioned differently. One scan was taken for each sample with an averaging time of 1.0 second, and the blank control spectrum (buffer only) was subtracted from each scan.

Also, a 10% propyl-modified wet sol-gel was prepared following the same procedure as above with one exception with the starting materials: 2.650 mL of TMOS was added to 0.350 mL of propylTriMOS. Then, 1.000 mL of TMOS/ propylTriMOS mix was added to each of the three 15-mL plastic tubes. The next steps were the same as the standard sol-gel preparation, procedure and testing given above.

2.4 Protein Concentration

Bovine Cu/Zn SOD (bSOD1) was used in this research as a reference. The protein concentration and stock dilution needed for a good CD signal were determined using bSOD1 since its molecular weight and protein concentration were similar to the WT hSOD1 and A4V. A 1: 20 dilution of the original protein stock of 10 mg/ml (in 10 mM Tris buffer, pH 7.0) was used since it produced a strong signal with little noise from the total absorbance. The concentration of bSOD1 was determined using the UV-Vis spectrophotometer and by applying the Beer-Lambert law:

$$A = \epsilon \times c \times l$$

Where

- A is the measured absorbance,

- ϵ ($\text{L mol}^{-1} \text{ cm}^{-1}$) is the extinction coefficient at some characteristic wavelength,
- l (cm) is the pathlength of the cuvette in which the sample is contained,
- c ($\text{L}^{-1} \text{ mol}$) is the concentration of the protein.

The concentration is calculated as follows:

$$c = (A \times \text{dilution factor} \times \text{MW}) / (\epsilon \times l)$$

For the specific case of bSOD1:

- $A = 0.143$ (measured value obtained from the UV-Vis spectrophotometer),
- $\epsilon = 10,300 \text{ M}^{-1} \text{ cm}^{-1}$ at 258 nm (value obtained from Roe et al., 1998),
- $l = 0.200 \text{ cm}$,
- dilution factor = 20,
- $\text{MW} = 32\,500 \text{ g/mol}$ (OxisResearch); is the molecular weight used to convert concentration to g / L.

For the specific case of hSOD1:

- $A = 0.498$ (measured value obtained from the UV-Vis spectrophotometer),
- $\epsilon = 10,800 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm (value obtained from Rodriguez et al., 2002),
- $l = 0.200 \text{ cm}$,
- dilution factor = 20,
- $\text{MW} = 32\,000 \text{ g/mol}$ (Valentine et al., 2005).

To determine how much protein to use in standard sol-gel samples, the following equation was employed:

$$(\text{Total volume of sol-gel } 7.75 \text{ mL}) / (20 \text{ dilution factor}) = 0.39 \text{ mL.}$$

The protein's volume was rounded to 0.40 mL. However, as the protein aged there was a need to increase the protein's volume to 0.60 mL (0.5 mg/ml or 15.6 μ M) (1/40 is needed in a 2 mm cuvette path, and then 1/20 dilution will be used in a 1 mm thick glass, as determined by the cassette spacing).

The aqueous protein / buffer solution must have a volume of 4.65 mL. So, if the protein's volume is 0.60 mL, then the buffer's volume (either Tris, pH 7.0 or KOAc, pH 5.7) must be 4.05 mL by subtraction.

2.5 Converting Raw CD Data to Mean Residue Ellipticity ([θ])

Ellipticity values, θ , obtained from the CD instrument in units of mdeg were converted to the mean residue molar ellipticity [θ] in $\text{deg} \times \text{cm}^2 \times \text{dmol}^{-1}$ by using the following formula:

$$[\theta] = (\theta \times \text{MW} \times 100) / (N_A \times d \times 1000 \times C_M)$$

$$[\theta] = (\theta \times \text{MW}) / (N_A \times d \times 10 \times C_M)$$

Where:

- θ is the measured ellipticity (CD data in mdeg),
- N_A is the number of amino acids per protein (153 x 2) for dimer SOD1,
- d is the path length in cm (0.1 cm for a glass sample),
- C_M is the molar protein concentration,
 - $C_M = (c \times \text{protein's volume used in sol-gel}) / \text{total volume of sol-gel}$,
where c is the protein stock concentration obtained from the UV-Vis

instrument, the protein's volume used in sol-gel is 0.40 or 0.60 ml, and 7.75 mL is the total volume of sol-gel.

- Conversion factors:
 - 100 is used to convert the molar concentration to $\text{dmol} \times \text{cm}^{-3}$,
 - 1000 is used to convert millidegree to degree.

2.6 Addition of Metals to hSOD1 and A4V

Dilute stock metal solutions of Cu^{2+} and Zn^{2+} sulfate were added to ensure that the hSOD1 and A4V were fully metalated. The protein concentration [hSOD1] was estimated as follow: Assume 10 g / L stock protein in 5mM KOAc, pH 5.5. $[\text{hSOD1}] = (10 \text{ g / L} \times 1000) / (32000 \text{ g/ mol}) = 0.31 \text{ mM}$. The 1000 factor was used to convert molarity to a millimolar scale. Next, 30 μL of 20 mM Zn^{2+} sulfate was added to about 10 g / L of stock solution of hSOD1, and was incubated at 4°C for a few hours. Then, 30 μL of 20 mM Cu^{2+} sulfate was added to the protein stock solution and incubated at 4°C for a minimum of 24 hours. Since Cu^{2+} has the tendency to occupy both the zinc and the copper binding sites, Cu^{2+} sulfate was added after the incubation with Zn^{2+} sulfate (Valentine et al., 1979).

CHAPTER 3

3.0 Results

3.1 First Aggregation Condition: Heat

First, the CD spectra of WT SOD1 encapsulated in standard sol-gel was compared with that of WT in solution at 25°C to determine if any structural change occurred due to encapsulation. The reference spectrum shown in all of the figures in this document is the native conformation of encapsulated WT incubated in Tris buffer, pH 7.0, at 2°C.

Similarly, for A4V figures, the reference used is that of the encapsulated A4V protein under like conditions. The reference of WT and A4V tests in solution is the native WT and A4V conformation in Tris buffer, pH 7.0, at 25°C. Based on the spectra shown in Figure 8A, the structural difference that could be attributed to encapsulation is minor (225-240 nm). While the sol-gel encapsulation process may trap proteins in non-native conformations as reported with hemoglobin (Khan et al., 2000) and apomyoglobin (Eggers and Valentine, 2001b), no such conformational changes were observed with WT or A4V SOD1 encapsulation.

Next, the effects of heat treatment on the sol-gel encapsulated WT protein were compared with that of WT in solution. The spectra shown in Figures 8B-E are remarkably similar, and the major improvement in thermal stability, as reported previously for other proteins (Eggers and Valentine, 2001a), was not observed in this case. The secondary structure of the sol-gel encapsulated sample at 85°C (Figure 8F) appears to be more denatured than the solution structure at 85°C. This may be due to the fact that solution spectra were taken immediately upon reaching the desired temperature,

whereas sol-gel samples were equilibrated for about 5 minutes at each temperature before scanning.

Figure 8. (A-I). Thermal denaturation of WT and A4V SOD1 at various temperatures. (A-F) CD spectra in the far-UV comparing WT SOD1 secondary structure in solution and in sol-gel at temperatures ranging from 25-85°C. The references shown for WT and A4V SOD1 are the spectra in solution without encapsulation, in Tris buffer, pH 7.0, at 25°C. (G-I) CD spectra in the far-UV comparing A4V SOD1 secondary structure in solution and in sol-gel at 25, 37, and 85°C.

Finally, the effects of heat treatment on the secondary structures of sol-gel encapsulated WT and A4V proteins are compared (Figure 9). The structure of encapsulated A4V was found to be extremely sensitive to heat treatment as compared with WT. The encapsulated A4V sample began to denature at 37°C as compared to 75-85°C for the WT (Figures 9B-C and 8G-I). In addition, the A4V sample denatured in two different structural conformations depending on the applied temperature range. From 37-55°C, A4V conformational changes were different than those in the range of 65-85°C. The more negative molar ellipticity around 220-240 nm and 202 nm regions, and the positive trend around 210-215 nm region, suggests that a decreased β -structure and an increased random coil structure has transpired. Protein damage with heat treatment in the above range was irreversible for both the WT and A4V. The thermal stability of the A4V protein was not tested in solution since this mutant is known to aggregate (Ray et al., 2004).

Figure 9. Thermal denaturation of SOD1. CD spectra of SOD1 in the far-UV showing the effect of heat from 25-85°C on (A) WT in solution (B) encapsulated WT in sol-gel, and (C) encapsulated A4V in sol-gel.

3.2 Second Aggregation Condition: Low pH

The effects of different low pH conditions (pH 4.0 and 5.7) on the secondary structure of the encapsulated WT and A4V proteins are examined in this section and compared to the structure at pH 7.0, which is used as a reference.

3.2.1 Encapsulated WT and A4V Proteins in Tris Buffer, pH 7.0

Initial expectations were that the encapsulated WT protein would be rather stable at pH 7.0. However, several drastic potential intermediate conformational changes were observed over the two-month test period, most of which occurred on the seventh and 11th day of the experiment (Figure 10A). The WT sample was stored at a constant 4°C during the entire test period, except when the CD measurements were taken at 25°C for short periods. A change in the secondary structure on the seventh day (Figure 10B) took place with a negative CD band at around 212 nm. In addition, this conformational change resulted in the loss of intensity of CD signal for all the tests that followed. This suggests that the unfolding/refolding process resulted in leaching of some of the encapsulated protein out of the sol-gel matrix. Furthermore, there is a strong evidence, as will be discussed in another section of this study, that the negatively charged protein at pH 7.0 (pI ~ 5.8) is repelled by the negatively charged sol-gel matrix. Moreover, leaching is dependent on the sol-gel pore size, which may differ for samples prepared with Tris pH 7.0, and pH 5.7 (Hoang and Lee, 1997). Again, this will be discussed in more detail in Chapter 4, but it is mentioned here mainly to explain why this leaching did not occur with the A4V sample shown in Figure 10. Hence, in order to compensate for the lower protein concentration and facilitate comparison with the encapsulated A4V under similar

conditions, the CD data is normalized to have the same ellipticity value near the minimum. This is shown in Figures 10B-B' and a similar compensation is performed for the final measurement on the 54th day (Figures 10C-C'). Based on the normalization factors used for the seventh and 54th day spectra, approximately 40% of the protein was lost after the seventh day, and an additional 10% was lost over the rest of the test period (11th – 54th days). It is important to note that the odd measurement taken on the seventh day cannot be due to protein leaching out of the sol-gel matrix since the CD signal intensity on the ninth and 11th days are more negative in the 200-220 nm region (Figure 10A). Thus, the CD data normalization shown in Figure 10-B' is performed mainly to highlight the remarkable similarity with the final A4V conformation in Figure 10E. Given that other odd measurements similar to the one observed on the seventh day are also noticed under different test conditions, this conformation could be a potential transient intermediate or simply an artifact. The possible causes are discussed in Chapter 4.

Figure 10. CD spectra in the far-UV of encapsulated SOD1 incubated in Tris buffer, pH 7.0. WT sample equilibrated (A) for 1, 2, 3, 7, 9, 11 and 54 days, (B) for 1, 2, 3, and 7 days, (B') compensation for signal in day 7 as observed in A and B, (C) incubation after 54 days and (C') compensation for signal in (C). (D) A4V sample equilibrated for 2, 4 and 54 days, and (E) after 54 days.

With the encapsulated A4V protein, a similar conformation to the one encountered with WT (seventh day) was observed, but the major difference is that the A4V protein did not refold to the native conformation even after 54 days (Figures 10B')

and E). In addition, the encapsulated A4V did not leach out of the sol-gel (*i.e.*, no loss of signal was encountered) under identical test conditions used with WT except for the sol-gel samples being prepared with slightly different methods (pH 7.0 vs. pH 5.7). This suggests that the A4V conformational changes were different as compared with the WT, but more importantly that these changes were irreversible. By comparing the spectra shown in Figures 10B'-C' and Figure 10E, the final A4V conformational change was obviously more similar to the possible transient intermediate WT change that took place on the seventh day, as compared to the final WT conformation. The positive CD signal in the 225-235 nm region could be attributed to either disulfides or aromatic side chains (Sreerama and Woody, 2004). Even though aromatic side chains affect the CD signal significantly in the above region, especially for proteins with low α -helix content (Sreerama and Woody, 2004), it is suspected that the disulfide bonds are the major contributors in this case in spite of the fact that SOD1 protein has less than 7% α -helix content. This is based on other tests shown in section 3.4 that specifically target the disulfide bonds (*i.e.*, addition of TCEP/DTT). It is important to note that the encapsulated A4V protein showed a significant dip in the 225-235 nm region (*i.e.*, more negative ellipticity) in all of the other test conditions except for this one. This region (225-235 nm) was unaffected even after 54 days of incubation at pH 7.0, but the entire CD spectrum appeared to be slightly red-shifted as a result. Finally, this region was more positive for A4V SOD1 as compared with WT SOD1 in both solution and sol-gel encapsulated measurements.

3.2.2 Encapsulated WT and A4V Proteins in KOAc Buffer, pH 5.7

The WT protein was encapsulated in two different standard sol-gel batches. In the first batch, KOAc buffer, pH 5.7, was used as the aqueous protein / buffer component during the sol-gel preparation, and in the second batch, Tris buffer at pH 7.0 was used. The protein stock and concentration used for both sol-gel batches was identical.

From the first batch (prepared with pH 5.7 buffer), a WT sample was incubated in KOAc buffer, pH 5.7, for 54 days at 4°C, and CD measurements were taken periodically. Figures 11A-C show all the conformational changes observed with this sample. The most notable change in the secondary structure occurred on the third day, which indicates less content of β -structure (Hornemann and Glockshuber, 1998; Sreerama and Woody, 2004; Fujiwara et al., 2005) with the characteristic 215 nm signal intensity change (Figure 11B). On the seventh day the β -structure content becomes close to the native structure. After 54 days of incubation the conformation has a more negative value at the minimum near 209 nm than the native structure (Figure 11C).

Figure 11. CD spectra in the far-UV of encapsulated WT SOD1 in two different sol-gel batches. A sol-gel sample prepared with KOAc buffer, pH 5.7, incubated in KOAc, pH 5.7, at 4°C is represented from (A-C); (A) incubation for 1, 2, 3, 7, 9, 11 and 54 days, (B) for 3, 7, 9 and (C) 54 days. A sol-gel sample prepared with Tris buffer, pH 7.0, incubated in KOAc, pH 5.7, at 4°C is described from (D-E); (D) incubation for 1, 2, 3, 7, 9, 11 and 54 days, (E) for 7 and (F) 54 days.

Surprisingly, the WT sample from the second batch (prepared with Tris, pH 7.0) demonstrated a completely different behavior under identical conditions (Figures 11D-F). None of the interesting potential transient intermediates that were observed with the other batch occurred with this sample. As will be discussed in Chapter 4, this is most likely due to the pore size difference of the sol-gel samples prepared with pH 7.0 and pH 5.7. Nonetheless, after 54 days of incubation, the change that occurred in the region between 200-210 nm (Figure 11F) suggested that the metals might have been released. To test this hypothesis, additional measurements were conducted on the same sample, where Zn

and Cu ions were added (Figures 12A-B). While the addition of Zn ion caused a significant transformation in the secondary structure, the addition of Cu ion did not result in any changes in the conformation. Recently, Potter and co-workers reported similar results with the addition of Zn ion to the apoprotein in solution (Potter et al., 2007). After metal addition, the sample was placed back in KOAc, pH 5.7, for 28 days, and the final conformation that resulted was a near-native structure with a decrease in the negative ellipticity in the region between 205-215 nm (Figure 12C). Finally, the same sample was put in KOAc, pH 4.0, to destabilize the native fold (Chitin et al., 2002). After one day of incubation in pH 4.0 at 4°C, no major change was observed, but, after only 10 minutes of incubation at 37°C, a notable change in the 200-215 nm region was detected (Figures 12D-E). The sample was then incubated at 37°C for longer periods resulting in a drastic change in the secondary structure (200-210 nm and 225-235 nm) (Figure 12F). This spectrum suggests that the protein's structure is largely unfolded at this stage. The dip in the 225-235 nm region is likely to be due to breaking the disulfides, to be discussed further in section 3.4. After another 20 days of incubation at 4°C in pH 4.0, the WT SOD1 appeared to regain structure, but the final conformation was a non-native structure (Figure 12G). It seems that the destabilization to the protein structure and the associated unfolding was partially reversible, unlike the temperature effects that were found to be irreversible. The WT SOD1 protein has shown an impressive tendency to fold and refold after partial unfolding under the effect of low pH at 37°C. This is in striking contrast to the A4V protein, as will be discussed in later sections.

Figure 12. CD spectra in the far-UV of encapsulated WT SOD1 under various conditions. (A) In KOAc, pH 5.7, for 54 days followed by Zn ion for 1 and 4 days, then Cu ion for one day, and then in KOAc, pH 5.7. (B) Addition of Zn ion for 4 days. (C) Incubation in KOAc, pH 5.7 for 28 days after the addition of Zn ion. (D) Equilibration in KOAc, pH 5.7, for 28 days was followed by incubation in KOAc, pH 4.0, for one day at 4°C, 10 minutes, and 3 days at 37°C, then 1 day at 4°C, another day at 37°C, and 20 days at 4°C, (E) effect of KOAc, pH 4.0, at 37°C for 10 minutes, (F) in pH 4.0, for one day at 37°C, and (G) the final conformation after 20 days in KOAc, pH 4.0, at 4°C.

Since none of the measurements that were observed with the second batch (sol-gel prepared with pH 7.0) showed the possible transient intermediates observed with the first batch (sol-gel prepared with pH 5.7), additional tests were conducted on the first batch

(pH 5.7). The same sample that was used previously (54 days in pH 5.7) was subjected to other conditions to see if the previously observed potential transients could be reproduced. The sample was first placed in Tris buffer, pH 7.0, and then changed to 40 mM TCEP. No CD spectrum was obtained due to the magnitude of noise caused by the high absorbance of TCEP. Consequently, the sample was placed in Tris buffer, pH 7.0, for 2 days at 4°C, and then a measurable CD signal was obtained. The conformational changes that resulted are shown in Figures 13A-B. The change in the secondary structure (Figure 13B) is close to that observed on the third day with the original sample (Figure 11B), however the protein leached out of the sol-gel matrix as shown in the following measurements (Figures 13C-D). In order to facilitate comparison with other measurements, the spectra were multiplied by a compensation factor as described previously to normalize the results (Figures 13C'-D'). The final conformation indicates that the protein did fold back to the native conformation, and the dip in the range between 200-208 nm is perhaps the result of releasing the metal in the process (by comparison to the apoprotein spectrum). EDTA was added at the end of this experiment but produced no further effects, suggesting that the metal had already been released in a previous step.

Figure 13. CD spectra in the far-UV of encapsulated WT SOD1 incubated in various buffers. (A) In KOAc at pH 5.7 for 54 days followed by Tris buffer, pH 7.0, after 1 day in TCEP, then another 6 days in Tris, pH 7.0, then placed back in KOAc, pH 5.7, for 2 days, and finally in 30 mM EDTA for 6 days, (B) in Tris, pH 7.0, after 1 day in TCEP, (C) followed by Tris, pH 7.0, for 6 days, KOAc, pH 5.7, for 2 days, and in 30 mM EDTA for 6 days; (C') compensation for protein loss in (C), (D) CD WT protein in 30 mM EDTA for 6 days and (D') compensation for protein loss in (D).

Based on the results obtained with WT, A4V protein was encapsulated in the standard sol-gel made with KOAc buffer, pH 5.7. After one day of incubation at 4°C,

there was a noticeable difference in the starting point of the A4V spectrum relative to the solution spectrum with an increase in negative ellipticity between 220-250 nm as shown in Figures 14A-B. Another A4V sample was tested to confirm the change in the secondary structure, and the new sample produced identical results (Figures 14C-E). Then, Zn^{2+} was added to determine if the dip between the region 250-220 nm is related to metal loss (Figure 14D), but no major change was observed. It is important to note that no potential transient intermediates with β -characteristics were detected with the mutant A4V protein in pH 5.7 or 7.0, as previously was shown with the WT protein.

Figure 14. CD spectra in the far-UV of encapsulated A4V SOD1 incubated in various buffers at 4°C. (A) First sample equilibrated in KOAc, pH 5.7, for 1, 2, and 12 days and (B) 12 days. (C) A second sample equilibrated in KOAc, pH 5.7, for 2 and 12 days followed by the addition of Zn ion for 7 days, and then replaced with Tris pH 7.0 for 29 days, (D) in KOAc, pH 5.7, for 12 days followed by the addition of Zn ions for 7 days, and (E) in Tris, pH 7.0, for 29 days.

3.2.3 Encapsulated WT and A4V Proteins in KOAc Buffer, pH 4.0

This section focuses on the destabilization effect of low pH on the secondary structure of WT and A4V SOD1. Four WT samples were used; three of which were encapsulated in sol-gel made with KOAc, pH 5.7, and the fourth was encapsulated in sol-gel made with Tris, pH 7.0.

The first WT sample (sol-gel made with pH 5.7) was incubated in 10 mM KOAc buffer, pH 4.0, at 4°C for 54 days (Figures 15A-C). Most of the observed structural changes occurred in the 200-210 nm region, but none of the β -characteristics that were observed with pH 5.7 were encountered (Figures 11B and 13B). After 54 days, there was an increase in the negative ellipticity between 200-210 nm and a minor change in 228-230 nm region indicating a slight destabilization of the protein. Since the secondary structure was stable at 4°C, a different approach was needed to perturb the structure and trap any intermediate conformations.

The second WT sample (sol-gel made with pH 5.7) was initially incubated in pH 4.0 at 37°C for 40 minutes to accelerate the destabilization, and then it was placed back at 4°C (Figure 15D-F). After one day at 4°C, a significant change was detected in the CD spectrum in the region between 200-210 nm (Figure 15E), and the signal resembled the apoprotein spectrum in solution (Potter et al., 2007). Thus it was assumed that metal loss occurred. After four days, further changes were detected in the region between 200-210 nm as well as 225-235 nm (Figure 15E). The sample was left in the same condition (at 4°C) for six additional days, and the final conformation on the 10th day (Figure 15F) shows that the WT protein reverted to a conformation somewhat resembling the reference

except for the dip in the region between 200-210 nm. In addition, the recovery in the region between 228-230 nm was rarely observed with WT protein and never with the A4V mutant.

The third WT sample (sol-gel made with pH 5.7) was incubated in KOAc, pH 4.0, at 37°C for multiple days. The protein adopted a random coil structure after just one day of incubation (Figure 15H). After four days, the structure unfolded completely (Figure 15I). Increasing the temperature from 4 to 37°C at pH 4.0 had a devastating effect on the stability and integrity of the WT structure.

Finally, the fourth WT sample (sol-gel made with pH 7.0) was incubated in KOAc, pH 4.0, at 4°C for 7 days (Figures 15J-L). After seven-day incubation, the protein signal resembles the WT sample encapsulated in sol-gel made with pH 5.7 under identical test conditions (KOAc, pH 4, at 4°C).

It may be worthwhile to note that with pH 4.0, no potential transient intermediates or folding/refolding of the protein were observed as compared with Tris, pH 7.0 and KOAc, pH 5.7. The main effect of lowering the pH to 4.0 is to destabilize the protein's native fold, which is a necessary process to begin aggregation (Chiti et al., 2002). This pH-induced unfolding is irreversible in the glass matrix if the temperature is raised to 37°C.

Two encapsulated A4V protein samples (sol-gel made with pH 5.7) were subjected to similar condition as the WT samples (10 mM KOAc buffer, pH 4.0, at 4 °C) for 28 and 20 days, respectively. The results were radically different than those observed with the WT samples.

The first A4V sample was incubated at 4°C for a total of 28 days (Figures 16A-C). After just one day of incubation, an increased negative ellipticity was observed in the spectrum region between 200-210 nm (Figure 16B). This change was more pronounced and slightly different than with the WT protein after two days at pH 4.0. Also, after two days of incubation, a drastic loss of the prominent ellipticity band at 230 nm was observed. This loss was common to the A4V protein under different aggregation conditions. Further unfolding continued after seven days, and then the structure became random coil after 28 days (Figure 16C).

The second A4V sample was subjected to low pH as above, but at 37°C (Figures 16 D-F). Initially the sample was incubated at 4°C for three days, which resulted in a similar destabilization as above. Consequently, the sample was subjected to heat treatment. After just 10 minutes at 37°C, there was a major impact on the secondary structure, and after 3 days at 37°C the protein was fully unfolded (Figure 16 E). The sample was then incubated for an additional 20 days at 4°C to see if the protein will attempt to refold, but the damage was irreversible (Figure 16 F).

The data suggests that the mutant A4V is very susceptible to irreversible unfolding in an acidic environment even at a low temperature, 4°C. Therefore, the use of less harsh conditions such as pH 5.7 and 7.0 might be able to slow down the unfolding

process in an attempt to detect any possible intermediates that might have been missed at pH 4.0.

Figure 16. CD spectra in the far-UV of encapsulated A4V SOD1 incubated in KOAc, pH 4.0. (A) The first sample for 28 days at 4°C, (B) 1, 2, 7 and (C) 28 days. (D). The second sample was incubated at 4°C for 3 days, 10 minutes and 3 days at 37°C, 2 days at 4°C, 1 day at 37°C and 20 days at 4°C, (E) effect of temperature at 3 days at 4°C followed by 10 minutes and 3 days at 37°C and (F) 20 days at 4°C.

3.3 Third Aggregation Condition: Removing Metals with EDTA

Since apo-SOD1 has been implicated in aggregation studies performed *in vitro*, understanding the behavior and structural changes of the apoprotein or the conditions that might lead to metal loss could shed some light on its possible role in aggregation. To this end, both WT and A4V proteins were equilibrated in EDTA at 4 and 37°C. All samples incubated in 10 mM EDTA exhibited a very high ratio of noise to signal in the far-UV CD spectrum thus making analysis of the data impossible. To solve this problem, the

samples were washed with purified water and incubated in 10 mM Tris buffer at pH 7.0 for about 30 minutes prior to testing in order to dilute the EDTA concentration and decrease the noise. This process does not affect the results because metal chelation by EDTA should have been complete after being equilibrated for 24 hours (Eggers and Valentine, 2001a).

A sol-gel encapsulated WT sample made with KOAc, pH 5.7, exhibited a potential transient intermediate conformation at 215 nm after two days of incubation in 10 mM EDTA, and then the protein reverted to the native conformation (Figures 17A-C). Interestingly, from the CD spectra there was no indication that an apoprotein was obtained during those three days, *i.e.*, there was no detection of an increased negative ellipticity in the region between 200-207 nm.

On the other hand, WT encapsulation in a sol-gel made with Tris, pH 7.0 did show possible metal loss (Figure 17E) after seven days in 10 mM EDTA. The CD spectrum showed an increased negative ellipticity in the region between 200-210 nm that suggested the formation of apoprotein. However, with the loss of metals, further destabilization of the native structure was expected to occur, but that was not the case. After 54 days, the CD signal resembled the reference with a near-native like conformation, but the signal in the 200-207 nm region was unusual (unlike native conformation or apoprotein). To determine if this was due to metal loss, Zn^{2+} was added after rinsing the glass to remove EDTA (Figures 17G-I). Even though, after the first day of adding Zn^{2+} , there was an apparent change in the CD spectrum, after the fourth day there was no significant change from the initial condition. Cu^{2+} was added next, even

though there is no direct evidence implicating its role in the structural stability. Again, no change was detected (Figure 17G). It is possible that with this altered near-native conformation, the Zn^{2+} could not be added, or a permanent damage occurred in the zinc-binding site, or the Zn^{2+} was added in a different region of the protein. Thus, the sample was placed back in EDTA and monitored for a long time (72 days) to determine which of the above possibilities is most likely. Given the lack of additional conformational changes (Figures 17H-I), the possibility of the zinc ion binding to a different location can be ruled out.

Figure 17. CD spectra in the far-UV of encapsulated WT SOD1 protein equilibrated in 10 mM EDTA at 4°C. (A) The first sample at day 1, 2 and 3; (B) at day 2 and (C) day 3. (D) The second sample incubated for 54 days, (E) day 1, 2, 3, 7 and (F) 54; (G) after 54 days in 10 mM EDTA, addition of Zn ion for 1 and 4 days, then Cu ion for 1 day, and then placed back in 10 mM EDTA for 72 days; (H) the addition of Zn ion for 1 and 4 days after incubation in 10 mM EDTA for 54 days, and then (I) the sample was placed back in 10 mM EDTA for 72 days.

Since the use of 10 mM EDTA interfered in the CD readings, to circumvent this problem a lower concentration of 0.33 mM determined by trials (protein concentration for the entire cassette was 0.5 mg/ml) was used in the following tests (Figures 18A-C).

After 10 days of incubation, the WT structure began to lose its native configuration accompanied by a decrease in negative ellipticity between the region 220-230 nm and 207-200 nm, which indicated metal loss (Figure 18C). When the incubation temperature was increased to 37°C, a change in the spectrum was observed after one day of incubation (Figures 18D-E). After two days, metal loss was observed (Figure 18E). After four days, metal loss appeared to be complete, as indicated by the loss in the secondary structure relative to the metallation reference spectrum in Potter and co-workers (2007) (Figure 18F).

Figure 18. CD spectra in the far-UV of encapsulated WT SOD1 as a function of 0.33 mM EDTA. (A) The first sample was incubated at 4°C for 1, 2, 3, 4 and 10 days, (B) for 3 and (C) 10 days. (D) The second sample was placed at 37°C for 1, 2, 3 and 4 days, (E) 1, 2 and (F) 4 days.

Encapsulated A4V protein that was incubated in 10 mM EDTA at 4°C showed an increased negative ellipticity in the region between 225-240 nm and 200-220 nm (Figures 19A-B). To confirm the possibility of metal loss, Zn^{2+} was added after removal of EDTA, and a corresponding change was detected in the 200-220 nm region (Figure 19B). When the sample was placed back in EDTA, a corresponding change was observed in the same region (Figure 19C). These changes suggest that the Zn^{2+} was added to the apo-A4V protein. However, the changes in the structure did not agree with the work of Potter and co-workers (2007) done in solution on apo-WT protein. Most likely mismetallation happened in the mutant A4V SOD1 because of the abnormalities in the metal-binding properties of the zinc-binding site due to the mutation (Valentine et al., 2005). It is important to note that changes in the CD signal observed between 225-240 nm were also encountered with other A4V signals under various conditions such as pH 4.0 and 5.7, and that the addition of metal does not have effects on that part of the spectrum.

Another A4V sample was used to confirm the demetallation effect under milder conditions (0.33 mM EDTA). After five days of incubation in 0.33 mM EDTA, a significant change was observed in the CD regions between 225-240 nm and 200-220 nm (Figures 19D-E). Then the temperature was raised to 37°C for 10 minutes, and the sample was measured. Next, the same sample was again incubated at 37°C for two consecutive 15-minute periods, and readings were obtained. The corresponding change in the secondary structure (Figure 19E) shows that the initial 10-minute period did most of the damage (increase in the negative ellipticity in the above mentioned regions). The complete unfolding of the structure took place after 28 days at 4°C (Figure 19F). These

observations also suggest the presence of apo-A4V protein. Finally, subjecting the encapsulated A4V sample to one day at 37°C in the presence of 0.33 mM EDTA produced irreversible unfolding of the secondary structure (Figures 19G-H).

Figure 19. CD spectra in the far-UV of encapsulated A4V SOD1 under the effect of EDTA. See legend on the following page.

Figure 19. Legend:

(A) The first sample was incubated in 10 mM EDTA at 4°C for 2 and 12 days followed by addition of Zn ion for 1 and 7 days then placed back in 10 mM EDTA for 29 days, (B) effect of Zn ion after 1 and 7 days on the CD signal of A4V protein incubated in 10 mM EDTA for 12 days, (C) after 29 days in 10 mM EDTA at 4°C. (D) The second sample incubated in 0.33 mM EDTA for 1 and 5 days at 4°C followed by 10, 15 and 15 minutes at 37°C, and then for 28 days at 4°C, (E) effect of temperature was monitored after 1 and 5 days at 4°C, then at 10, 15 and 15 minutes at 37°C and (F) after 28 days at 4°C. (G) The third sample was placed in 0.33 mM EDTA at 37°C for 1 day, then at 4°C for 2, 6, 28, and (H) 28 days.

3.4 Fourth Aggregation Condition: Reducing the Disulfide Bond Using DTT or TCEP

Two reducing agents were used, namely DTT and TCEP, to characterize the effect of disulfide bond reduction on both the WT and A4V proteins. Literature reports provided contradicting results: Arnesano and co-workers (2004) stated that reducing the disulfide bond should have no effect on the secondary structure of WT protein; however Fujiwara et al. (2005) showed some loss of the structure using CD. It is expected that upon reducing the disulfide bond a general destabilization of the secondary structure would occur. In this work, it was found that slight variations in the test conditions influenced the secondary structure of the encapsulated reduced SOD1. The effects of TCEP are studied first, followed by DTT. The samples incubated in 40 mM TCEP and 6 mM DTT were washed with purified water and incubated in 10 mM Tris buffer at pH 7.0 for about 30 minutes prior to testing in order to decrease the noise level.

Initially, a WT SOD1 sample was reduced using 40 mM TCEP, pH 5.5, and stored at 4°C (Figures 20A-C). The results seemed to agree with Arnesano and colleagues (2004). There was a minor change in the 200-205 nm range, which was not significant enough to justify a new finding. On the other hand, when reduction took

place at 37°C, the WT protein unfolded within one day of incubation (Figures 20D, E). It seems that increasing the temperature enabled the structural changes in reduced WT protein. In addition, when two other older samples, previously used to study the long-term effects of pH 5.7 and pH 4.0, were subjected to TCEP treatment, major secondary structural changes were noticed in Figures 20F-H and Figures 20I-K, respectively. The reduced secondary structure under the effect of pH 4.0 became more random coil.

Figure 20. CD spectra in the far-UV of encapsulated WT SOD1 as a function of TCEP treatment. See legend on the following page.

Figure 20. Legend:

(A) The first sample was incubated in 40 mM TCEP at 4°C and readings were obtained for 1, 2, 3, 4 and 10 days; (B) 2 and 4 days and (C) 10 days. (D) The second sample was incubated at 37°C, and readings were monitored for 4 consecutive days with (E) showing day 4. (F) The third sample was previously in KOAc at pH 5.7 for 54 days at 4°C, and reduced with 40 mM TCEP for 1 day, then placed in KOAc, pH 5.7 for 2 days, put in Tris, pH 7.0, for 6 days and then placed back in KOAc, pH 5.7 for 2 days; (G) reduced WT protein in KOAc, pH 5.7, for 2 days; (H) effect of KOAc, pH 5.7, for 2 days after incubation in Tris, pH 7.0, for 6 days. (I) The fourth sample previously placed in KOAc, pH 4.0, for 54 days at 4°C, was reduced with 40 mM TCEP for 1 day and then put in Tris, pH 7.0, for 2 and 6 days, and finally incubated in KOAc, pH 4.0, for 6 days; (J) in KOAc at pH 4.0 for 54 days, then placed in Tris at pH 7.0 for 2 days after 1 day in 40 mM TCEP, then left in Tris, pH 7.0 for 6 days, and (K) placed in KOAc, pH 4.0 for 6 days.

To study the effects of DTT on the secondary structure, three WT samples encapsulated in sol-gel made with KOAc, pH 5.7, were reduced with 6 mM DTT, pH 5.5, and stored at 4°C. Then, each sample was washed with purified water and then placed in a different buffer, at pH 7.0, 5.7 and 4.0, to monitor the effect on the secondary structure (Figures 21A-I). In buffer of physiological pH, the reduced WT protein maintained the native structure (Figures 21A-C). Also, in pH 5.7 buffer, no major changes occurred in the secondary structure (Figures 21D-F). However, at pH 4.0 the reduced WT protein went through a conformational change, exhibiting a CD signal around 215 nm that is reminiscent of a β -structure. After two weeks, the protein refolded to an altered conformation similar to the reference, but with a major difference in the CD region between 210-200 nm (Figures 21G-I). It is important to note that the previous oxidized WT samples that were subjected to pH 4.0 at 4°C did not show the major structural change in the reduced WT sample shown in Figure 21H. This suggests that even though reducing the disulfide bond did not produce an apparent change in the secondary

structure, something else may have changed in order for the reduced protein to produce different results when placed in pH 4.0. Perhaps future studies could investigate these further using techniques other than far-UV CD that was solely used in this study. One possible method could be a fluorescent quenching technique to study modifications in the protein conformation (Pierre, 2004).

Figure 21. CD spectra of encapsulated WT SOD1 following DTT treatment. See legend on the following page.

Figure 21. Legend:

(A) The first sample was in 6mM DTT for 2 days, then in Tris, pH 7.0, for 4 days; (B) showing spectrum in 6 mM DTT for 2 days and (C) in Tris, pH 7.0, for 4 days. (D) The second sample was in 6 mM DTT for 2 days followed by KOAc, pH 5.7, for 4 and 18 days; (E) in 6 mM DTT for 2 days, and (F) in KOAc, pH 5.7, for 18 days. (G) The third sample was in 6 mM DTT for 2 days followed by KOAc, pH 4.0, for 4 and 18 days; (H) in KOAc, pH 4.0 for 4 days, and (I) for 18 days.

Next, the effects of TCEP on encapsulated A4V protein were investigated. Three samples were used. Two samples were treated with TCEP first, and then put in different buffers, one in Tris, pH 7.0, and the other in KOAc, pH 5.7. The third sample was initially placed in KOAc, pH 4.0, and then treated with EDTA. The TCEP concentration was 40 mM at pH 5.5 for all the samples.

The samples that were initially treated with TCEP showed a similar destabilization to the secondary structure (Figure 22). Also, the subsequent pH condition at pH 4.0 did show additional deterioration indicating a possible metal loss. To confirm this, EDTA was added to the sample that was in pH 4.0 (Figures 22G-I), and the resulting signal (Figure 22H) was nearly identical, thus confirming that the metal loss occurred in a prior step. This loss of metals could be a consequence of the destabilization in the A4V protein due to the mutation, even though Hough and co-workers indicated that the metal binding sites were unaffected by the mutation (Hough et al., 2004). However due to the destabilization in the dimer interface caused by the mutation of alanine to valine, unfolding or metal loss *in vivo* may result (Hough et al., 2004). Finally, the A4V protein unfolded (Figures 22G-I) after seven days at pH 4. This unfolding was irreversible since the sample was monitored for an additional 16 days with no change in the structure.

The third sample that was initially placed in pH 5.7, then in TCEP, showed similar initial deterioration, but after five days in TCEP, the metal loss was not observed, although the protein fully unfolded after 29 days in TCEP (Figures 22D-F).

Finally it is important to note that the encapsulated oxidized A4V protein incubated in pH 7.0 buffer did not exhibit similar changes in the secondary structure, even after 54 days of incubation (Figure 10E), as compared to the reduced encapsulated A4V protein in pH 7.0 (Figures 22A-C).

Figure 22. CD spectra of encapsulated A4V SOD1 as a function of TCEP treatment. See legend on the following page.

Figure 22. Legend:

(A) The first sample was incubated in 40 mM TCEP for 2 days, then in Tris, pH 7.0, for 1 and 6 days; (B) in 40 mM TCEP for 2 days followed by Tris, pH 7.0 for 1 and (C) 6 days. (D) The second sample was in KOAc, pH 5.7, for 2 days then in 40 mM TCEP for 5 and 29 days; (E) in KOAc, pH 5.7, for 2 days followed by 40 mM TCEP for 5 days, and (F) in 40 mM TCEP for 5 and 29 days. (G) The third sample incubated in 40 mM TCEP for 2 days followed by KOAc, pH 4.0, for 5 days, 30 mM EDTA for 5 days and then in KOAc, pH 4.0 for 7 and 16 days; (H) in 40 mM TCEP for 2 days, KOAc, pH 4.0 for 5 days and 30 mM EDTA for 5 days; (I) in KOAc, pH 4.0 for 5 days and 16 days.

The CD spectra of reduced A4V protein, with 6 mM DTT, pH 5.5, at 4°C, again showed an increased negative ellipticity between 250-220 nm (refer to Figures 23A-F). Similar observations can be made in this section to those described with the prior TCEP section. These results are in line with previous work done with SOD1 in solution (Tiwari and Hayward, 2003; Fujiwara et al., 2005). Furthermore, the same CD regional change mentioned above was also observed when A4V protein was incubated under other destabilizing conditions such as pH 4.0 or addition of EDTA. This common change in the CD spectra could be related to the A4V mutation, which decreases the global stability of the protein (Hough et al., 2004). The A4V protein was shown to be more susceptible to disulfide reduction than WT SOD1 protein in the present work.

Figure 23. CD spectra of encapsulated A4V SOD1 following treatment with DTT. (A) The first sample was incubated in 6mM DTT for 4 days, then in Tris, pH 7.0, for 2 and 16 days, (B) in Tris, pH 7.0, for 2 and (C) 16 days. (D) The second sample was equilibrated in 6 mM DTT for 4 days followed by KOAc, pH 5.7, for 2, 16 and 50 days, (E) in KOAc, pH 5.7, for 2, and (F) 16 and 50 days. (G) The third sample was in 6 mM DTT for 4 days followed by KOAc, pH 4.0, for 2 and 16 days, (H) in KOAc, pH 4.0 for 2, and (I) for 16 days.

3.5 Fourth Aggregation Condition: TFE and Methanol

The denaturant effects of alcohols such as 2,2,2-trifluoroethanol (TFE) on protein structure originate from the alcohol's low polarity, which is believed to interfere with the hydrophobic forces stabilizing protein structure through the alcohols direct interactions

with water (Voet and Voet, 2004). Furthermore, TFE has been shown to induce a helical conformation in an all β -barrel protein (Srisailam et al., 2003). Also, methanol can cause the formation of helical structure or β -like structure (caused by aggregation of the helical intermediate form) depending on the specific protein or polypeptide (Wang et al., 1992). Therefore encapsulated WT and A4V proteins were tested with various concentrations of TFE and methanol to investigate the effect of these denaturants on the secondary structure of SOD1. All samples were encapsulated in sol-gel glasses made with KOAc, pH 5.7. A4V samples that were previously exposed to different conditions were replaced with various concentrations of TFE and methanol.

Three different WT glass samples were used. The first one was incubated in 15% TFE at 4°C for four consecutive days. A CD reading was obtained for each incubation day (Figures 24A-C). A change in the CD signal in the region around 208 nm was detected indicating an increase in the helical content of the structure (Sreerama and Woody, 2004). The next two samples were used to compare the effects of TFE and methanol on WT secondary structure. The two samples started with identical initial conditions, *i.e.*, physiological pH and then 15% TFE at 4°C. When TFE concentration and temperature were increased to 80% at 37°C, the secondary structure in the first sample unfolded within one day (Figures 24D-F). Interestingly, when methanol was added instead of TFE to the second sample, the secondary structure was largely restored to its native conformation (Figures 24G-I). This recovery of the native conformation has not been observed before.

Figure 24. CD spectra in the far-UV of encapsulated WT SOD1 due to the effect of TFE and methanol. (A) The first sample was placed at 4°C in 15% TFE for 1, 2, and 4 days, (B) for 1, 2, and (C) 4 days. (D) The second sample was in Tris, pH 7.0, for 1 day followed by 15% TFE at 4°C for 5 days then in 80% TFE for 1 day at 37°C, (E) in 15% TFE at 4°C for 5 days, and (F) in 80% TFE for 1 day at 37°C. (G) The third sample equilibrated in Tris, pH 7.0 at 4°C for 1 day, 15% TFE at 4°C for 5 days then in 80% MeOH for 1 day at 37°C, (H) in 15% TFE at 4°C for 5 days, and (I) in 80% MeOH for 1 day at 37°C.

Two samples were used to study the effect of TFE and methanol on the secondary structure of mutant A4V protein. The first sample was previously incubated in Tris, pH 7.0, at 4°C for two months. The near-native conformation was documented in Figures

10D-E. Addition of TFE was performed to determine its effects on the A4V protein exhibiting the stable near-native conformation since TFE has been shown to induce aggregation in SOD1 (Stathopoulos et al., 2003). Upon the addition of 15% and then 45% TFE at 37°C, an increase in negative ellipticity was detected in the CD regions around 208 nm (similar to WT SOD1) and 225-235 nm. So, by increasing the TFE concentration, A4V protein began to unfold (Figures 25A-C). The second experiment used an A4V protein sample incubated in Tris, pH 7.0 for one month at 4°C. The CD spectrum showed the loss of the prominent band in the region 228-230 nm, which is a common feature observed in A4V protein under various aggregation conditions, *i.e.*, low pH with KOAc pH 5.7 and 4.0, (sections 3.2.2 and 3.2.3), EDTA (section 3.3), and TCEP/DTT (section 3.4). When TFE was added at 4 and 37°C, no change in the secondary structure was observed (Figures 25D-E). When low concentration of methanol (15%) was added, the CD signal intensity decreased indicating leaching of some of the encapsulated protein out of the sol-gel matrix (Figure 25F). The third experiment used a reduced A4V protein sample. When various concentrations of TFE were added at 4 and 37°C, similar results were obtained as with the two previous experiments (Figures 25G-H). When 80% methanol was added at 37°C, the A4V protein adopted a new conformation with a distinct minimum in the CD region at around 215 nm as compared to 208 nm in the native structure (Figure 25I). This minimum has been observed before with the WT spectra but not with the A4V spectra. Further research needs to be done using new samples of encapsulated A4V SOD1 to better understand the effects of TFE and methanol.

3.6 Other Test Conditions

3.6.1 Effects of Salts on the Secondary Structure of hSOD1

Some salts are known to have stabilizing effects on protein structure while others have the opposite or no such effect (Jain and Ahluwalia, 1996). In this work, $N(CH_3)_4Cl$ (TMACl) was used to study its effects on the encapsulated hSOD1 proteins. This salt was selected since it has been shown to have stabilizing effects on encapsulated proteins that were denatured either due to the encapsulation process or through intentional chemical denaturing (Eggers and Valentine, 2001b). In addition this salt exhibited minimum interference with the CD spectrum in the far-UV region, which makes it a perfect candidate for this study.

Two encapsulated WT samples and one A4V sample were incubated in 1.0 M $N(CH_3)_4Cl$, pH 7.0 and stored at 4°C. The first WT sample was encapsulated in a standard sol-gel made with KOAc buffer at pH 5.7 (Figures 26A-C), and the second sample was encapsulated in a sol-gel made with Tris buffer, pH 7.0 (Figures 26D-F'). Both samples were monitored for 54 days. A4V protein was encapsulated in a standard sol-gel made with KOAc buffer, pH 5.7 (Figures 26G-H), and the sample was monitored for 2 days. The spectra shown in Figures 26A-H indicate that $N(CH_3)_4Cl$ had a destabilizing effect on the secondary structure of the encapsulated protein. This was surprising given that previous studies have shown opposite effects. The possible causes for this apparent conflict are discussed in detail in Chapter 4.

The final conformations of the encapsulated proteins are compared in Figure 27 after normalization. The loss of the CD signal in the case of WT protein encapsulated with sol-gel made with Tris buffer at pH 7.0, as shown in Figures 26D-F', is suspected to

be mainly due to the pore size being larger as compared with the pore size of the sol-gel made with pH 5.7 buffer. This is consistent with other CD measurements observed in this study as will be discussed in section 3.6.2. The minor normalization for the A4V signal was necessary due to the length of the incubation period (2 vs. 54 days) rather than the pore size. This, unfortunately, was needed since the sample broke after the second measurement. Interestingly, the CD spectra of the encapsulated A4V and WT proteins were almost identical in this case, whereas in most other test conditions different effects were observed on the encapsulated A4V and WT proteins.

Figure 26. CD spectra in the far-UV of encapsulated WT and A4V SOD1 protein equilibrated in 1M N(CH₃)₄Cl. See legend on the following page.

Figure 26. Legend:

(A) The first WT sample was encapsulated in standard sol-gel made with KOAc buffer, pH 5.7 and monitored for 1, 2, 3, 7, 9, 11 and 54 days, (B) 7 and 11 days and (C) 54 days. (D) The second WT sample was encapsulated in standard sol-gel made with Tris buffer, pH 7.0 and monitored for 1, 2, 3, 7, 9, 11 and 54 days, (E) 7 and 11 days and (F) 54 days. (D', E', and F') are normalized for D, E and F respectively. (G) A4V sample encapsulated in standard sol-gel made with KOAc buffer, pH 5.7 and monitored for 1, 2 and, (H) 2 days.

Figure 27. Comparison of final conformations of encapsulated WT and A4V SOD1 incubated in 1.0 M $N(CH_3)_4Cl$. A4V sample encapsulated in standard sol-gel made with KOAc buffer, pH 5.7 was incubated for 2 days is compared to WT samples incubated for 54 days (in sol-gel made with KOAc buffer, pH 5.7 and with Tris buffer, pH 7.0 normalized signal). Both WT and A4V references are shown.

3.6.2 Effect of pH 8.0 on the Encapsulated hSOD1

Protein leaching out of the sol-gel pores presented a major problem in this research. It was noted that as the pH increased from 5.7 to 7.0, so did the leaching from the sol-gel. This loss could be attributed to the electrostatic interaction between the sol-gel matrix and the protein. Human SOD1 is predicted to have an isoelectric point (pI) around 5.8, hence at pH 7.0 and above the protein carries a net negative charge (Shaw

and Valentine, 2006). In addition, the silica pI is about 2.1, so at higher pH the silica walls become negatively charged (Nguyen et al., 2002) and that would create an electrostatic force that would repel the protein from the pores. To confirm this observation, two encapsulated WT samples and one encapsulated A4V sample were incubated in Tris buffer, pH 8.0 at 4°C for 54 days and 12 days, respectively. As will be discussed further in chapter 4, the net charge of the protein in pH 8.0 buffer is expected to be significantly more negative as compared with that in pH 7.0 (-7.3 and -3.2, respectively). The low temperature (*i.e.*, 4°C) slowed down the leaching process and allowed careful monitoring of the loss rate. The two WT samples were prepared using the standard sol-gel protocol. One was made with Tris buffer, pH 7.0 (Figures 28A-A') and the other was made with KOAc buffer, pH 5.7 (Figures 28B-B'). Similarly, the A4V sample was prepared using the latter method (Figures 28C-C').

First, the general trend is established by comparing the spectra shown in Figure 11, Figures 10A-C, and Figures 28A-B corresponding to WT samples incubated in different buffers with pH 5.7, pH 7.0 and pH 8.0, respectively. This confirmed that as the pH increased, the protein signal decreased. Similarly, for the encapsulated A4V sample, by comparing the spectra shown in Figure 28C and Figures 14A-B, an analogous trend is observed. In order to determine the rate of protein leaching due to the above observations, the normalization factors used to achieve the same ellipticity value near the minimum are used to calculate the loss percentage under various conditions. As shown in Figure 29, the rate of protein leaching correlates with the buffer pH as well as the sol-gel preparation method. Under identical incubation conditions, the extent of leaching of

SOD1 encapsulated in standard sol-gel glasses made with KOAc buffer at pH 5.7 is found to be always lower than that encapsulated in sol-gel glasses made with Tris buffer at pH 7.0. This suggests that the pore size is smaller for the sol-gel prepared with pH 5.7 as compared with pH 7.0. In addition, the pH of the buffer used for incubation appears to have a more significant effect on SOD1 leaching rate as shown in Figure 29.

Figure 28. CD spectra in the far-UV of encapsulated WT and A4V SOD1 incubated in Tris pH 8.0 at 4°C. (A) WT sample was encapsulated in standard sol-gel made with Tris buffer, pH 7.0 and monitored for 1, 2, 3, 7, 9, and 54 days. (B) WT sample was encapsulated in standard sol-gel made with KOAc buffer, pH 5.7 and monitored for 1, 2, 3, 7, 9, and 54 days. (C) A4V sample encapsulated in standard sol-gel made with KOAc buffer, pH 5.7 and monitored for 1 and 12 days. (A', B', and C') are normalized for A, B and C respectively.

Figure 29. Effects of buffer pH and sol-gel preparation methods on encapsulated protein-leaching rate.

3.6.3 Bovine SOD1 (bSOD1) Tests under Various Conditions

Parallel tests were conducted using bSOD1, rather than human WT or A4V, in order to establish test guidelines and to avoid the potential waste of the limited amount of WT and A4V proteins available for this study. These tests were primarily focused on addressing the following: 1) Effects of the sol-gel encapsulation on the protein's secondary structure as compared with that in solution when incubated in pH 7.0 buffer. 2) Stability of the encapsulated protein under various test conditions and determination of how long the protein structure remains stable in the encapsulated environment. 3) Effects of the different types of sol-gel glasses used in this study (*i.e.*, 100% TMOS and 10% propyl-modified glass). 4) Changes in the secondary structure of the protein under the

influence of various pH, salts, and other aggregation conditions such as low pH, EDTA, and heat.

First, the CD spectrum of native bSOD1 in solution (10 mM Tris, pH 7.0) was obtained and compared with the literature (Foti et al., 1997). Both signals were similar, and the spectrum obtained was used as the bSOD1 reference for all the following tests (Figure 30). Next, two different encapsulation processes were used to determine the impact on the secondary structure. The first encapsulation process was based on the standard sol-gel method described in (Eggers and Valentine, 2001a), and the buffer used was Tris, pH 7.0. The second process included a 10% propyl-modified sol-gel. As shown in Figure 30, the encapsulation processes produced minor effects on the secondary structure of bSOD1 protein.

Figure 30. Effects of the encapsulation process on bSOD1. The first sample was encapsulated with a standard sol-gel protocol, and the other sample with a 10% propyl-modified sol-gel protocol. Both samples were incubated with Tris buffer, pH 7.0, at 4°C. bSOD1 in solution was used as the reference.

Protein leaching out of the sol-gel pores, causing loss of the CD signal, was a major problem for this protein. To circumvent this issue, sol-gel samples were used

immediately after the two weeks of maturation, and samples were kept at 4°C except during CD measurements, which were conducted at 25°C unless stated otherwise. As observed in Figure 31, most of the samples had a sufficient amount of protein to produce a good CD signal even after 12 days of incubation, although the samples incubated in 10 mM Tris buffer at pH 8.0 and $N(CH_3)_4Cl$ were weaker. The two different encapsulation processes also produced similar spectra (Figures 31A-F' and Figures 31I-I') except for $N(CH_3)_4Cl$. The effects of $N(CH_3)_4Cl$, as described previously, are discussed further in Chapter 4. Finally, the denaturation effects resulting from heat treatment, as shown in Figure 31H, are not only irreversible, but in fact appear to continue further when the sample is stored at 4°C after treatment.

The effects of the different encapsulation processes on the secondary structure of bSOD1 are compared under various incubation conditions (Figures 32A-C'). Again no major difference is observed aside from the $N(CH_3)_4Cl$ case, which is the same data shown in Figures 31F-F'.

Figure 31. CD spectra in the far-UV of encapsulated bSOD1 incubated in various conditions. See legend on the following page.

Figure 31. Legend:

Samples in spectra from (A-H) were encapsulated in a 10% propyl-modified glass, and incubated for 12 day at 4°C with the exception of the sample in (G-H). (A). Samples were incubated in Tris buffer, pH 7.0, (B). in KOAc buffer, pH 5.7, (C). in KOAc buffer, pH 4.0, (D). in EDTA, (E). in Tris buffer, pH 8.0, (E'). Normalization of (E), (F). in $N(CH_3)_4Cl$ and (F'). Normalization of (F). (G). bSOD1 sample was incubated in Tris, pH 7.0, for 1 day and then heated from 25-95°C, afterward stored at 4°C, and readings were taken after, (H). 4, 6 and 12 days. (I). CD readings were obtained of samples encapsulated in the standard sol-gel and incubated for 1 day at 4°C in Tris, pH 7.0 and 8.0, KOAc, pH 5.7 in EDTA and $N(CH_3)_4Cl$. bSOD1 in solution was used as the reference.

Figure 32. Comparison of CD spectra in the far-UV of encapsulated bSOD1 using the standard sol-gel and the 10% propyl-modified glass in various buffers. All samples (A-C') were incubated for 1 day at 4°C. (A). Samples were incubated in Tris, pH 8.0, (B) in KOAc, pH 5.7, (C) in $N(CH_3)_4Cl$, and (C') Normalization of (C). bSOD1 in solution was used as the reference.

3.6.4 Other Test Conditions: Xerogel

Encapsulation of WT SOD1 in xerogel was investigated under five conditions. While samples incubated in Tris buffer, pH 7.0, KOAc, pH 5.7 and EDTA unfolded, the sample incubated in $N(\text{CH}_3)_4\text{Cl}$ did not (Figures 33A-A'). Given that in the xerogel protocol more shrinkage and water loss occurred as compared with the standard wet-aged sol-gel process, the resulting average pore sizes are assumed to be much smaller (Pierre, 2004). This could contribute significantly to the loss of the secondary structure of the protein due to the presence of small amount of entrapped water surrounding the protein in the pores as well as the silica matrix interaction (Eggers and Valentine, 2001a, 2001b). The beneficial effects of $N(\text{CH}_3)_4\text{Cl}$ in this case are discussed in Chapter 4.

Figure 33. CD spectra in the far-UV of encapsulated hSOD1 WT in xerogel. (A). Samples were incubated in Tris buffer, pH 7.0, KOAc buffer, pH 5.7, EDTA and TMACl for 1 day at 4°C. (A') normalization of (A). The reference is a sample of WT encapsulated in standard sol-gel incubated in Tris buffer, pH 7.0, at 4°C.

CHAPTER 4

4.0 Discussion and Conclusions

This study demonstrates that the encapsulation of CuZnSOD proteins (human WT, mutant A4V, and bovine) in sol-gel matrices is a viable approach to isolate intermediates that would normally lead to aggregation in dilute solutions. The findings in this study are pertinent from two perspectives. First, since protein aggregation in solution is inevitable under various denaturing conditions, establishing the effects of such conditions on the structure of these proteins is difficult, if not impossible, in some cases. This is especially true for the mutant SOD1 A4V protein since it has been shown to aggregate spontaneously *in vitro* under conditions at which the WT protein remains stable (Ray et al., 2004). Second, the effects on protein structure due to molecular crowding and confinement in the cellular environment are mimicked by protein encapsulation in the sol-gel (Eggers and Valentine, 2001a). Given that most of the previous studies on SOD1 have been conducted in dilute solutions, understanding the influence of the highly crowded cellular environment on protein stability and dynamics is of great interest (Zimmerman and Minton, 1993; van den Berg et al., 1999; Ellis, 2001; Zhou and Dill, 2001). The secondary structure of the entrapped proteins was investigated using circular dichroism in the far-UV region (200-250 nm). Near-UV measurements and tertiary structure impact were out of scope in this study given the limited amount of protein available (*i.e.*, protein concentration in the samples should be at least five times that used in this study). Various sol-gel processes were investigated as described below, and two types of wet-aged sol-gels were found to be most suited for hSOD1 entrapment with low

protein concentration. Finally, the influences of several denaturing conditions on the secondary structure of entrapped proteins are explored further in this chapter, and the most important finding of a “stabilized partially unfolded state” will be discussed at the end of this chapter.

4.1 Impact of the Various Sol-gel Methods on the Structure of Encapsulated Proteins

In this section, general findings related to the effects of sol-gel encapsulation, pore sizes, entrapped water and pH are discussed.

4.1.1 Sol-gel Methods: Summary and Findings

Four sol-gel preparation methods were investigated in order to determine the impact of encapsulation on protein structure as well as to find a way to limit the protein leaching problem that plagued the initial tests. The silica sol preparation methods used in this study were based on the standard protocol described in the literature (Ellerby et al., 1992; Eggers and Valentine, 2001a). These methods are enumerated in the following with the associated impact on protein structure and leaching.

1. Standard wet-aged gels: The silica sols were prepared using two different buffers, namely, Tris, pH 7.0, and KOAc, pH 5.7. For brevity, the resulting glasses containing the entrapped proteins are referred to, hereinafter, as sol-gel^{7.0} and sol-gel^{5.7}, respectively. WT SOD1 was encapsulated using both gel types, whereas the mutant A4V protein was encapsulated only in sol-gel^{5.7} due to the limited amount of protein available. Bovine SOD1 was encapsulated using sol-gel^{7.0} and other sol-gel

types as described below. In the absence of denaturant, protein encapsulation in these glasses was found to induce a very minor change to the protein's structure as compared with that in solution at pH 7.0 at 25°C (Figure 8A). However, protein leaching was higher with sol-gel^{7.0} as compared with sol-gel^{5.7}, particularly when the glasses were incubated in buffers with a pH higher than the protein's pI ~ 5.8 (*i.e.*, when the encapsulated protein is negatively charged). In the presence of denaturants, the encapsulated proteins in these different glasses exhibited slightly dissimilar structural changes under identical incubation conditions with few exceptions. The most probable cause for this, as discussed in section 3.6.2, is that the average pore size of sol-gel^{7.0} is larger than sol-gel^{5.7}. This would explain both the higher protein leaching observed with sol-gel^{7.0} as well as the slight difference in the structural changes of the encapsulated proteins under various incubation conditions.

2. Xerogel: This method was considered for two reasons: 1) it offered a potential means to eliminate the protein leaching problem; 2) xerogels are less brittle than wet-aged sol-gels that broke more often than desired. Unfortunately, the resulting CD signal intensity was extremely weak, and the protein denatured significantly. While in a previous study, bovine SOD1 was successfully encapsulated in a xerogel and remained active (Ellerby et al., 1992), the concentration used in that study was about forty times that of the concentration used in this study (0.65 mM vs. 0.0156 mM). Thus, the higher concentration was not an option for this study based on the

required amount of protein and the fact that the absorbance would be too high for CD measurements in the far-UV.

3. Propyl modified glass: The addition of the propyl groups to the silica matrix was of interest based on the results reported in previous studies (Pierre, 2004; Rocha and Eggers, 2007); however, the encapsulation of SOD1, using this method, resulted in leaching of the protein. Consequently, the CD signal intensity was very weak in comparison with the 100% TMOS (standard) glass even when an identical protein concentration was used in the preparation. Therefore, with the 10% propyl-modified glass, the protein concentration needed in the sample should be more than doubled that of the standard glass to compensate for the leaching problem. In addition, the physical nature of the 10% propyl-modified glass samples was found to be extremely brittle. This resulted in a large percentage of samples breaking due to handling (*i.e.*, removal from the quartz cuvette for storage and the reverse process). As a result, aside from the bSOD1 encapsulation tests shown in section 3.6.3, this method was not preferred for hSOD1 encapsulation.

Thus, most of the experiments in this study used the standard wet-aged sol-gel for encapsulating both WT and A4V SOD1. The protein leaching issue was partly addressed by using sol-gel^{5,7} for most of the tests, as well as storing the samples at 4°C, which was found to lower the leaching rate further. Lastly, in cases where protein leaching could not be avoided (*i.e.*, when the pH buffer was higher than the protein's pI 5.8), CD data normalization was used to compensate for the resulting loss of protein concentration (*i.e.*, approximate the signals at the same concentration).

4.1.2 Impact of Sol-gel Pore Size on Encapsulated Protein Structure

Since the average pore size of the different glasses appears to have a definite impact on the protein leaching at elevated pH (section 3.6.2), the associated impact on protein folding was investigated. Comparison of the CD spectra of encapsulated WT SOD1 in the different glasses under various incubation conditions reveals that there is a definite impact in most cases except one, namely, with $\text{N}(\text{CH}_3)_4\text{Cl}$. As shown in Figures 11, 15, and 17, corresponding to incubation in KOAc buffer at pH 5.7, pH 4.0, and in EDTA, respectively, the intermediate and final structural changes were different. In the case of $\text{N}(\text{CH}_3)_4\text{Cl}$, it is suspected that the additional hydrophobic interactions predominate over the electrostatic forces (Jain and Ahluwalia, 1996) resulting in identical protein conformation. This will be discussed further in section 4.2. In the case of the xerogel, it was expected that the protein conformational change would be different than the wet sol-gel due to the smaller pore size. However, the data gathered in this case is considered unreliable due to the limited protein concentration used as discussed previously, and the resulting signal to noise ratio. On the other hand, the xerogel data could be viewed as a very rough estimate. With that in mind, the data show that there is a definite difference in protein structure when incubated in $\text{N}(\text{CH}_3)_4\text{Cl}$ as compared with other buffers (section 3.6.4, Figure 33) regardless of the sol-gel method used for encapsulation.

4.1.3 Effect of Water Confinement in the Silica Pores

While there is certainly no shortage of theories and hypotheses regarding the critical role that water plays on protein folding, many of the physical properties of water itself remain elusive (Stanley et al., 2007). Nevertheless, recent experiments have

revealed an intriguing water property when confined in silica nanotubes made with MCM-41 with pore diameter of 15 ± 1 Å (Liu et al., 2007, Stanley et al., 2007). Such confinement changed the water behavior significantly such that water remained in the liquid state when supercooled down to 160 K (-113°C). In other studies, different aspects of confinement on water structure and dynamics are discussed. Takamuku and colleagues (1997) showed various water structure and dynamic properties depending on water confinement in porous silica of two different pore sizes (30 and 100 Å). Furthermore, the vibrational energy of confined water molecules in porous silica, with pore size of 90 Å at ambient temperature, is proven to be lower than that of bulk/unconfined water (Hall et al., 1981).

The observations above suggests that the loosely defined “bulk water” should not be assumed to possess the same properties as the “confined bulk water” within the silica pores, and that the properties of the confined water is also critically dependent on the level of confinement (*i.e.*, water properties could be slightly different in pore sizes of 15, 30, and 100 Å). The relevance of these observations to this study is that the conformational changes noted in the previous section relating to pore sizes may not only be due to the obvious variable restriction on the rotational movement of the protein, but that the change in water properties in these pores is an important factor. The average pore size for the gel^{7.0} is assumed to be around 100 Å (Eggers and Valentine, 2001a), but the reduction of the average pore size with sol-gel^{5.7} is unknown. However, considering the dimensions of the encapsulated SOD1, and considering that the encaging silica matrix around the protein does not form a perfect sphere, the amount of water entrapped

between the surface of the protein and the silica structure could be different even with a small change in the average pore size.

In addition to the water structure factor, there are other important considerations resulting from encapsulation such as the electrostatic interaction between the protein and the silica matrix. In particular, under most test conditions the active site of SOD1 is positively charged whereas the silica walls are negatively charged (pI around 2.5). This may not only be a factor after the encapsulation process is complete, but may play a role in the formation of the silica matrix around the protein. For instance, with the sol-gel^{7.0} protocol, while the net charge of the protein is negative, the active site is still positive while other parts of the protein's surface are negatively charged. Hence the formation of the silica matrix around the protein may favor interaction with certain parts of the protein's surface but not others. This is illustrated in Figure 34 by calculating the electrostatic potential around the WT SOD1 at pH 7.0 using DeepView/Swiss-pdb Viewer V3.7. The protein's data used for the model was obtained from the Protein Data Bank (PDB, ID 2C9V).

Figure 34. Electrostatic field around the SOD1 dimer at pH 7.0. Calculations were done using DeepView/Swiss-pdb Viewer V3.7. Protein data was obtained from PDB, ID 2C9V.

4.1.4 The Effect of pH 8.0 on Leaching

As discussed in section 3.6.2 in Figure 29, there was a significant increase in protein leaching when incubated in pH 8.0 as compared with pH 7.0 buffers. This was surprising since the encapsulated protein did not show a major change in the secondary structure, which could have easily been explained by the change in the net charge of the protein (*i.e.*, more residues become solvent exposed). Furthermore, this observation was consistent with hSOD1 (WT and A4V) and bSOD1 using different sol-gel protocols. This increase in the net negative charge is obviously due to more residues on the protein becoming deprotonated with pH 8.0, but which residues was unclear. To address this, the

following two basic questions are considered: which residues are the potential suspects, and are these residues exposed, or partially exposed, to solvent in the native fold?

First, all of the 153 amino acids per WT SOD1 subunit are listed, and the frequency of each is plotted as shown in Figure 35. Among these, the polar-charged basic or acidic residues are highlighted. The pK_R of the side chains of both lysine and arginine are above 10, and are therefore always positively charged at pH 7.0 and 8.0. Aspartate and glutamate have pK_R around 4, thus will be negatively charged in the above pH range. This leaves the amino acid residues histidine and cysteine as the major suspects with pK_R of 6.04 and 8.37, respectively. Using the Henderson-Hasselbach equation, the fraction of the His and Cys side chains carrying a positive charge vs. pH is plotted as shown in Figure 36. From the figure, 90% of His side chains are deprotonated at pH 7.0, and an additional 9% will be deprotonated at pH 8.0. Similarly, for Cys an additional 26% will be deprotonated at pH 8.0. However, this assumes that the His and Cys residues are solvent exposed and there are no interactions with other residues. As a result, the next step is to look at where the His and Cys residues are located on the protein and their states.

Figure 35. Plot of all the amino acids found in a WT SOD1 subunit. Polar acidic and basic amino acid residues are negatively and positively charged, respectively. The blue color indicates the positively charged amino acid residues, and the red color the negatively charged amino acid residues at pH 7.0. The percentage of each amino acid residue out of the 153-total is shown at the bottom.

Figure 36. Fraction of histidine and cysteine side chains carrying a positive charge at different pH values.

Out of the eight His amino acids present per SOD1 subunit, six (His⁴⁶, His⁴⁸, His⁶³, His⁷¹, His⁸⁰ and His¹²⁰) are involved in the copper and zinc metal binding site, and two are not (Goto et al., 2000). The remaining two are: His⁴³, which is buried in the β -strand 4 and thus inaccessible, and His¹¹⁰ is solvent exposed as shown in Figure 37 (PDB: 2C9V and MBT Protein Workshop were used to generate the views). In addition, out of the four Cys residues, two form the disulfide bond (Cys⁵⁷ and Cys¹⁴⁶), Cys⁶ is buried, while Cys¹¹¹ is solvent exposed. As a result, in the fully metallated dimer, two His¹¹⁰ and two Cys¹¹¹ residues could be deprotonated further at pH 8.0. However, it should be noted that this estimate does not take into account the pK shifts related to the folded protein. The protein's net charge under different pH values was estimated by using a public tool (<http://www.scripps.edu/cgi-bin/cdputnam/protcalc3>), as shown in Appendix-A. Based on the output, the protein's net charges are estimated to be -4.2 and -7.3 at pH 7.0 and 8.0, respectively. This difference may be significant enough to justify the major increase in protein leaching observed at pH 8.0 due to increased charge repulsion.

Figure 37. Structural views of holo-WT SOD1 subunit showing the positions of the solvent exposed His¹¹⁰ and Cys¹¹¹. (Figure from PDB, ID 2C9V, 180° rotation)

4.2 $N(CH_3)_4Cl$ Effect on Encapsulated SOD1

The effect of $N(CH_3)_4Cl$ on the secondary structure of entrapped SOD1 was found to be denaturing to the native conformation with one exception, namely, the xerogel encapsulation. This was surprising given that this salt has been shown in previous studies to produce a stabilizing influence on the structure of various proteins in aqueous solutions

(lysozyme) as well as proteins entrapped in sol-gel glass (apomyoglobin and serum albumin) (Jain and Ahluwalia, 1996; Eggers and Valentine, 2001a, 2001b). Since this apparent contradiction may be due to several factors, a number of tests are compared below to determine the possible causes. Another intriguing observation was that the denatured structure for both the entrapped WT and A4V SOD1 appeared to be almost identical, which was a rare exception in this study. Furthermore, the conformation appeared to be a “stabilized” partial unfolding that was noticed with the WT SOD1 using different denaturing conditions. This will be discussed further at the end of this section, but first the apparent inconsistency with the studies listed above needs to be addressed.

In this study, six different sol-gel samples were subjected to $\text{N}(\text{CH}_3)_4\text{Cl}$ treatment as summarized in Table 2 below. The denaturation influence of $\text{N}(\text{CH}_3)_4\text{Cl}$ was confirmed with all but the xerogel case, where the effect appeared to be stabilizing (Figure 33). Also it is important to note that the xerogel encapsulation process denatured the protein significantly, and in that case the addition of $\text{N}(\text{CH}_3)_4\text{Cl}$ countered the denaturing influence of the silica matrix. Thus, this part is consistent with previous studies (Eggers and Valentine, 2001a, 2001b). However, with other sol-gel methods the encapsulation process did not denature the protein’s native fold and the effect of $\text{N}(\text{CH}_3)_4\text{Cl}$ in this case was denaturing. This destabilizing effect of $\text{N}(\text{CH}_3)_4\text{Cl}$ was also observed in solution by Koga and co-workers (2006). These results suggest that the additional hydrophobic or electrostatic interactions introduced by the addition of $\text{N}(\text{CH}_3)_4\text{Cl}$ are not necessarily always stabilizing (Jain and Ahluwalia, 1996).

Table 2. Summary of $N(CH_3)_4Cl$ effect on SOD1 using different sol-gel preparation methods.

	Sol-gel preparation protocols			
	sol-gel prepared with KOAc pH 5.7 (gel ^{5.7})	sol-gel prepared with KOAc pH 7.0 (gel ^{7.0})	10% Propyl modified glass	Xerogel
Effect on entrapped WT-hSOD1	Destabilizing the initial native fold, but new conformation is stable. (Figure 26C)	Destabilizing the initial native fold, but new conformation is stable (Figures 26F-F')	not tested	Stabilizing (Figure 33)
Effect on entrapped A4V-hSOD1	Destabilizing the initial native fold, but new conformation is stable (Figure 26H)	not tested	not tested	not tested
Effect on entrapped WT-bSOD1	not tested	Destabilizing the initial native fold, but new conformation is stable (Figures 32C-C')	Destabilizing the initial native fold, but new conformation is stable (Figures 32C-C')	not tested

In addition to the above, the different results observed with this and previous studies may involve other factors relating to the type of proteins used. For instance, by comparing the secondary structure of SOD1 with the two proteins used in previous studies, namely, apomyoglobin and serum albumin (Eggers and Valentine, 2001b), the following differences are revealed: a) both apomyoglobin and serum albumin proteins are mostly alpha helical (75% and 72% helical content, respectively); b) SOD1 proteins have a very small alpha helical content (only 7% helical, 39% beta sheets, and 54% random coil); and c) the percentage of hydrophobic residues on the surface of the proteins may be another factor that is relevant in this case. Furthermore, by comparing different older

studies (von Hippel and Wong, 1965; Jain and Ahluwalia, 1996) relating to the influence of $\text{N}(\text{CH}_3)_4\text{Cl}$ on different proteins in solutions reveals that the results are not consistently stabilizing either. Based on Jain and Ahluwalia, (1996) the thermal denaturation stability (T_m) of Lysozyme protein in aqueous solutions varies depending on $\text{N}(\text{CH}_3)_4\text{Cl}$ concentration. At high concentration (5-6 M), $\text{N}(\text{CH}_3)_4\text{Cl}$ demonstrates stabilizing influence on the protein's thermal stability, but at concentrations below 4 M $\text{N}(\text{CH}_3)_4\text{Cl}$ the opposite is true *i.e.*, $\text{N}(\text{CH}_3)_4\text{Cl}$ is indeed destabilizing. On the other hand, it was shown that the thermal stability of ribonuclease protein improves with $\text{N}(\text{CH}_3)_4\text{Cl}$ concentration of 0.5- 3 M (von Hippel and Wong, 1965).

In summary, the effects of $\text{N}(\text{CH}_3)_4\text{Cl}$, stabilizing or otherwise, are dependent on various factors including the protein type, $\text{N}(\text{CH}_3)_4\text{Cl}$ concentration, initial folding state, and the crowding environment.

4.3 The Effect of $\text{N}(\text{CH}_3)_4\text{Cl}$, pH 4.0 and TCEP on the Secondary Structure of Encapsulated SOD1

The effects of $\text{N}(\text{CH}_3)_4\text{Cl}$ on the entrapped SOD1 (WT and A4V) were found to be remarkably similar even when the encapsulation processes were different *i.e.*, using sol-gel^{7.0} and sol-gel^{5.7}. As mentioned previously, it is suspected that the additional hydrophobic interactions and/or the influence of $\text{N}(\text{CH}_3)_4\text{Cl}$ on the bulk water predominate over the pore size difference in this case. Intriguingly, the conformational changes for both the entrapped WT and A4V proteins appeared to be in a “suspended” partially unfolded state even after 54 days of incubation. In other words, the unfolding of

the encapsulated proteins was somehow halted, and the resulting denatured state was stable. Thus the key points that this section will focus on are what stabilized the protein in this state, and is there a different path to this state.

First, the CD spectra of this “suspended” partially unfolded state (*i.e.*, $N(CH_3)_4Cl$ measurements) are compared with other test data, and several conditions shown in Figure 38A appeared to produce similar CD spectra after normalization. The first comparison shows the CD spectrum of encapsulated WT-hSOD1 when incubated in pH 4.0 at 4°C for 54 days (Figure 15C), and the second is that of encapsulated A4V SOD1 under similar conditions but after only 2 days (Figure 16A). While the encapsulated WT SOD1 was stable after 54 days, A4V SOD1 under identical conditions continued to a complete and irreversible unfolding (Figure 16C). Furthermore, the encapsulated WT SOD1 sample described in section 3.2.2 (Figure 12G) appeared to refold to this “suspended” state even after being exposed to various buffers over 110 days. Finally, similar conformational changes were observed with EDTA (Figures 17I and 18C) implying metal loss.

The above mentioned confirmations, at least in the 225-235 nm region, appeared also to be somewhat similar to the CD spectra of encapsulated WT-hSOD1 intermediate on the way to complete unfolding after TCEP treatment at 37°C (Figure 20D) after normalizing. Because this implied a potential reduction of the disulfide bond, the two samples that were previously incubated in the $N(CH_3)_4Cl$ and KOAc (pH 4.0) were incubated afterwards in 40mM TCEP buffer for one day. The results demonstrated a slight structural change in the 225-235 nm and 200-208 nm regions (Figure 38B). Thus in order to determine if this minor difference was indeed due to a slight structural change

and in order to rule out potential measurement errors, the effects of TCEP and DTT on WT SOD1 in solutions were compared with the CD spectra of encapsulated proteins. The effect of TCEP on WT SOD1 in solution was investigated as part of this study, but the effect of DTT in solution was based on a previous study (Fujiwara et al., 2005) as shown in Figure 38C. The results were consistent, confirming that both TCEP and DTT had a slightly different impact on the protein's secondary structure as compared with the effects of $N(\text{CH}_3)_4\text{Cl}$ and KOAc at pH 4.0.

Figure 38. CD spectra of “suspended” partial unfolding for WT and A4V SOD1 under different conditions. (A). The first WT sample was incubated in TMACl for 54 days at 4°C, the second sample in pH 4.0 for 54 days at 4°C, and the third sample was incubated in TCEP for 1 day at 37°C. The first A4V sample was incubated in TMACl for 2 days at 4°C, the second sample in pH 4.0 for 2 days at 4°C, and the third sample in TCEP for 2 days at 4°C. (B). WT sample was incubated in TMACl for 54 days, then washed and replaced with TCEP. Another WT sample was incubated in pH 4.0 for 54 days, then washed and replaced with TCEP. (C). The first WT incubated in TCEP after TMACl, the second WT sample was equilibrated in TCEP after incubation in pH 4.0, the third WT sample in solution incubated in TCEP, and last WT sample incubated in DTT in solution taken from Fujiwara et al., 2005.

From the above, it is clear that the disulfide bond was being impacted by incubation in either $N(\text{CH}_3)_4\text{Cl}$ or KOAc pH 4.0 buffers, given that the change in the region between 225-235 nm was typically associated with the disulfides (Sreerama and

Woody, 2004). Metal loss was another possibility given the similarity with the EDTA CD spectra. However, this raises two more questions: 1) Why is there a difference in the 225-235 nm spectra in the TCEP test as compared with the $N(CH_3)_4Cl$ and pH 4.0 tests? 2) Because the encapsulated WT proteins completely unfolded under the influence of either TCEP or EDTA and similar complete unfolding was observed in solution, what stabilized the unfolding process?

One hypothesis was the formation of a non-native intramolecular disulfide bond involving Cys⁶ and Cys¹¹¹. This would not only stop the unfolding and stabilize the intermediate as observed but would also produce a slight difference in the CD spectra in the 225-235 nm region. In a recent study, Wang et al., (2006) suggested such a possibility as quoted below:

Although we focus discussion on intermolecular disulfide bonds, it is important to note that non-native intramolecular bonds could also occur, that is bonds involving Cys residues other than the native bond between C57 and C146, and that such bonds could stabilize non-native conformations. Hence, non-native disulfide bonding could play a role in both misfolding of the monomer and stabilization of multimers.

There was evidence of such scrambling of disulfide linkage involving Cys¹¹¹ (non-native) and Cys¹⁴⁶ (native) that was noticed with apo-SOD1 in vitro after an extended storage period at 4°C (Di Noto et al., 2005). In addition, other recent studies also implicated Cys¹¹¹ in the formation of intermolecular disulfide cross-linking leading to aggregation (Banci et al., 2007; Cozzolino et al., 2008). The creation of intermolecular disulfide links with the sol-gel-entrapped proteins is very unlikely due to the silica matrix. However, the structural changes that lead to the formation of intermolecular

bonds in solution could also produce non-native intramolecular disulfide bonds in the encapsulated environment. This hypothesis, of course, requires further validation, perhaps by repeating the experiment and doing additional testing in the near UV in the CD to obtain information regarding the tertiary structure and the disulfide bond. While other explanations for the above “suspended”, partially unfolded intermediate are possible, the non-native disulfide bond hypothesis is the most plausible candidate.

4.4 Effects of TFE and Methanol on Encapsulated SOD1

Before discussing the effects of alcohols used in this research, it is important to note that the A4V samples were previously incubated and tested in the various conditions listed in section 3.5 Figure 25. Thus the results observed could reflect the additive effects of multiple solution exchanges.

As observed in section 3.5, low TFE concentration increased the helical content in the WT as well as A4V SOD1 secondary structure. When the TFE concentration and temperature were increased to 80% and 37°C, the WT protein unfolded. On the other hand, when 80% methanol at 37°C was added to the partially unfolded WT protein, a recovery of the secondary structure was detected. Under the same conditions, the partially unfolded, reduced A4V protein exhibited a new secondary structure with an intense minimum at around 212 nm (as compared to 208 nm), and the absence of the peak in the region of 225-235 nm. These data raise an interesting question: Why did the SOD1 behave differently under the effects of these alcohols?

One possible answer is based on the physiochemical properties of the alcohols and their acidity compared to water. TFE with a pKa of 12.4 was more acidic than water (pKa of 15.7). From infrared and NMR spectroscopies, it was found that the increased acidity could decrease the fraction of water molecules with nonbonded lone pairs (Symons, 1981). Therefore, the increased acidity of TFE made water “less hydrogen-bond competent” (Kentsis and Sosnick, 1998). As a result, the occurrence of hydrogen bonding between amides and carbonyl moieties on the SOD1 and TFE was probably diminished, and that could lead to desolvation of the protein. On the other hand, methanol with a pKa of 15.0 was less acidic than TFE, and at high concentration had the opposite effect to TFE; it allowed WT protein to refold to the native conformation most likely due to the strong intramolecular hydrogen bonds in the protein main chain. Recently, it was observed that at high methanol concentration of 80%, as the hydrogen bonds between the protein and the solvent became weaker, the intramolecular hydrogen bonds in the protein main chain became stronger (Yamazaki et al., 2006). So the solvent acidity could change the properties of water, which in turn influence hydrogen bond formation and indirectly the hydrophobic interactions.

4.5 Metallation State of Encapsulated SOD1

Determining the metallation state of the encapsulated proteins purely based on the far-UV CD spectra proved to be more challenging than initially thought. This was primarily due to the fact that the CD region of interest (*i.e.*, below 210 nm) was rather noisy as shown in this study as well as in a previous work on SOD1 in solution (Fujiwara

et al., 2005). Despite this, when the encapsulated protein was incubated for longer periods, certain trends were clear and the CD signal became less noisy over time. The CD spectra for the encapsulated apo-WT SOD1 and holo-WT SOD1 were in agreement with the spectra obtained in solution (Arnesano et al., 2004; Potter et al., 2007). The major difference, however, was that the remetallation of the entrapped apo-WT SOD1 in sol-gel matrices was not always successful depending on various factors, whereas the entrapped apo-A4V SOD1 could not be remetallated at all. In the following, the experiment in which apo-WT SOD1 could not be remetallated is discussed first, followed by the successful case.

When incubated in EDTA for 54 days, the encapsulated WT SOD1 showed two conformational changes (Figures 17D-F). In the first seven to nine days, the conformational changes appeared to be very similar to the solution study (Potter et al., 2007). However, after a total of 54 days of incubation in EDTA, a new conformational change was observed that resembled the aforementioned “suspended” partial unfolding state. After forming this conformation, remetallating the entrapped protein was not possible, even though in solution studies it had been shown that both metals, Zn^{2+} and Cu^{2+} , could be added to the apoprotein (DiDonato et al., 2003; Potter et al., 2004). It was known that, upon metal loss, structural changes took place in the apoprotein; both the zinc and the electrostatic loops became highly disordered, and the zinc-binding site became solvent exposed. Yet, in this configuration the Zn^{2+} could still be added to the binding site (Banci et al., 2003). It was suspected, therefore, that the metal binding site of the entrapped protein was affected by the final conformation. Based on the CD spectra

shown in Figures 17F-I, the dip in the 225-235 nm region was also indicative of disulfide bond modification as discussed in the previous section. Thus the long-term incubation in EDTA (*i.e.*, 54 days) was suspected to induce not only metal loss in the first seven to nine days (Figure 17E), but also disulfide modification thereafter (Figure 17F). One possible explanation for this was that after the metal loss, the intra-subunit disulfide bonds were reduced resulting in two entrapped monomers, which then recombined to form the non-native intramolecular disulfide bond. Under similar conditions in solution studies (*i.e.*, when both metal loss and disulfide reduction occurred), the resulting monomers were shown to create intermolecular disulfide bonds (Banci et al., 2007). However, as mentioned in the previous section, the entrapped monomers were prevented by the silica matrix from forming intermolecular disulfide bonds, thus the formation of non-native intramolecular disulfide bonds was the most probable option. This could be an important finding for future studies since the remetallation of this species was not possible, and that could lead to loss of the enzymatic activity (*i.e.*, due to permanent metal loss) as well as the possibility of an early step in the aggregation pathway.

On the other hand, remetallating encapsulated WT SOD1 was possible under different incubation conditions. After incubating WT SOD1 in KOAc buffer at pH 5.7 for 54 days, the observed final conformational change was again similar to the apo-WT protein obtained in solution (Potter et al., 2007), and almost identical to the initial conformation noticed with EDTA (*i.e.*, 7-9 days) as shown by comparing Figure 11F with Figure 17E. It is important to note that wet sol-gel^{7.0} was used for both of the samples used in the previous EDTA experiment and in the one shown in Figure 11F. Thus the

sol-gel pore size or the encapsulation process variation was eliminated as a variable in this comparison. It was not clear why the protein lost the metal in this case but based on the CD spectrum, the data certainly implied that possibility. In order to confirm this, Zn^{2+} was added to the same sample, and the structural changes observed in Figure 12B (200-208 nm) were found to be similar to those observed with solution study for holo-WT and apo-WT respectively. The conclusion derived from this experiment is that remetallation of encapsulated apo-WT in sol-gel is possible, and that the failure of remetallation in the previous test with EDTA is not due to the sol-gel encapsulation but the structural changes to the protein.

4.6 WT-SOD1: Possible Intermediates or Artifacts

Out of the 660 CD measurements taken in this study, eight measurements showed abnormal CD spectra that were difficult to reproduce. These measurements were initially believed to be due to test errors or equipment glitches and thus discarded. However, later analysis revealed non-random patterns that could be due to a possible intermediate or an artifact. The observed secondary structure changes in these measurements were all red shifted with respect to the native fold, and the extent of the red shift seemed to correlate to the sol-gel process used for encapsulating the proteins as shown in Figures 39 and 40. With the sol-gel^{5.7} process, the CD minimum was consistently shifted from 208 to around 215 nm, whereas with the sol-gel^{7.0} process the minimum was shifted to around 212 nm. These red shifts were observed only with the entrapped human and bovine WT-SOD1, but not with the entrapped A4V-SOD1. Considering the number of CD measurements

taken during this study (*i.e.*, 180 for A4V, 450 for WT, and about 30 for the bovine SOD1), it was highly unlikely that these measurements represented random artifacts. In addition, the fact that these anomalous measurements were taken from five different sol-gel batches and that the extent of the red-shift correlated with sol-gel preparation method reinforced the possible link to the sol-gel process. However, whether these measurements represented possible ephemeral intermediates or artifacts related to the sol-gel process were difficult to determine. To address this, one of the CD measurements was repeated immediately after noticing the red shift (Figures 40F-F'). In this case the sample was left in the CD equipment and the measurement was repeated, within five minutes, without any modifications to the test conditions. As shown in the figure, the second measurement appeared to be native-like after normalization as compared with the first. While this strongly suggested the possibility of a sol-gel related artifact, the possibility of an ephemeral protein intermediate sol-gel related artifact could not be precluded since these experiments were not repeated due to the limited protein/glass samples. Glass sample-related artifacts may arise from a rough or inhomogeneous glass surface facing the light source of the CD spectrometer.

Figure 39. CD spectra of possible transient intermediates or artifacts with sol-gel^{5.7}. (A-B) Same as Figures 11A-B, human WT-SOD1, encapsulated in sol-gel^{5.7}, batch#1. (C-D) Same as Figures 13A-B, sol-gel sample reused. (E-F) Same as Figures 17A-B, different sample is used from the same batch as above. (G-H) Same as Figures 21G-H, different sample used from the different batch.

Figure 40. CD spectra of SOD1 as possible transient intermediates or artifacts with different sol-gel methods. (A-B) Same as Figures 31F, F'. (C-D) Same as Figures 31I, I'. (E-F) Same as Figures 26D-E'. (G-H) Same as Figures 10A, B. B', D', E', and F' are normalized spectra of B, D, E, and F, respectively.

4.7 Concluding Remarks

In conclusion, SOD1 entrapment in wet-aged sol-gel is proven to be a viable approach to obtain unique data that is not possible with solution tests in many cases. It is hoped that future investigations relating to SOD1 conformational changes will find the above data useful. The sol-gel encapsulation could be extended to other

neurodegenerative disorders where aggregation is an issue such as Alzheimer's, Parkinson's, Huntington's and prion diseases.

Important findings were as follows.

1. Sol-gel encapsulation

- The pH used during the encapsulation process affected the sol-gel pore size, protein leaching, and protein conformations.

2. Wt protein

- The salt $\text{N}(\text{CH}_3)_4\text{Cl}$ caused the protein to adopt a stable partially unfolded secondary structure under near physiological conditions (*i.e.*, pH 7.0 and crowding). Similar structural changes were also observed under two other conditions (*i.e.*, pH 4.0 and in TCEP/DTT).
- After 54 days of incubation in KOAc, pH 5.7, loss of Zn^{2+} ion occurred in the absence of a metal chelator.
- The uptake of the Zn^{2+} ion by the apo-WT SOD1 was not always possible.

3. A4V protein

- The secondary structure of the encapsulated A4V SOD1 exhibited a common increase in negative ellipticity in the region between 225 and 240 nm of the CD spectrum under the following aggregation conditions: KOAc, pH 5.7, EDTA, and TCEP/DTT.

Future studies to be considered.

- Testing the encapsulated WT protein using various salts (*i.e.*, other than $\text{N}(\text{CH}_3)_4\text{Cl}$) to determine if some of the salts that are abundant in human cells can cause the aforementioned stable partial unfolding.
- Further analysis of the stable partial unfolding state using a different testing method than CD such as the fluorescent quenching technique, to study modifications in protein conformation.

Appendix A: Protein Charge

Tool used to estimate protein charge at different pH values.

<http://www.scripps.edu/cgi-bin/cdputnam/protcalc3>;

PDB: 2c9v sequence used for input.

PROTEIN CALCULATOR v3.3

Accepted Sequence

ATKVVCVLKG DGPVQGIINF EQKESNGPVK VWGSIKGLTE GLHGFHVHEF

GDNTAGCTSA 60

GPHFNPLSRK HGGPKDEERH VGDLGNVTAD KDGVADVSIE DSVISLSGDH

CIIGRTLTVH 120

EKADDLGKGG NEESTKTGNA GSRLACGVIG IAQ 153

Estimated charge at pH 7.00 = -4.2

WARNING: pI estimate assumes all residues have pKa values that are equivalent to the isolated residues. For a folded protein this is **not** valid. However, this rough value can be useful for planning protein purifications. pKa values for the individual amino acids from Stryer Biochemistry, 3rd edition.

Estimated charge over pH range

pH	Charge
4.00	17.1
4.50	11.3
5.00	6.0
5.50	2.8
6.00	0.6
6.50	-1.9
7.00	-5.9
7.50	-9.9
8.00	-13.9
8.50	-16.4
9.00	-18.9
9.50	-21.3
10.00	-23.8

Both pH 7 and pH 8 are highlighted in green to show the estimated protein net charge, which is relevant to section 4.1.4.

Works Cited

- Andersen, P. M. Amyotrophic lateral sclerosis associated with mutations in the CuZn superoxide dismutase gene. *Curr. Neurol. Neurosci. Rep.* **2006**, 6, 37-46.
- Andrus, P. K.; Fleck, T. J.; Gurney, M. E.; Hall, E. D. Protein oxidative damage in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J. Neurochem.* **1998**, 71, 2041-2048.
- Arnesano, F.; Banci, L.; Bertini, I.; Martinelli, M.; Furukawa, Y.; O'Halloran T. V. The unusually stable quaternary structure of human Cu,Zn-superoxide dismutase 1 is controlled by both metal occupancy and disulfide status. *J. Biol. Chem.* **2004**, 279, 47998-48003.
- Banci, L.; Bertini, I.; Boca, M.; Girotto, S.; Martinelli, M.; Valentine, J. S.; Vieru, M. SOD1 and amyotrophic lateral sclerosis: Mutations and oligomerization. *Plos One* [Online] **2008**, 3, e1677. doi:10.1371/journal.pone.0001677.
- Banci, L.; Bertini, I.; Cramaro, F.; Del Conte, R.; Viezzoli, M. S. Solution structure of Apo Cu,Zn superoxide dismutase: Role of metal ions in protein folding. *Biochemistry* **2003**, 42, 9543-9553.
- Banci, L.; Bertini, I.; Durazo, A.; Girotto, S.; Gralla, E. B.; Martinelli, M.; Valentine, J. S.; Vieru, M.; Whitelegge, J. P. Metal-free superoxide dismutase forms soluble oligomers under physiological conditions: a possible general mechanism for familial ALS. *Natl. Acad. Sci. U.S.A.* **2007**, 104, 11263-11267.
- Borchelt, D. R.; Lee, M. K.; Slunt, H. S.; Guarnieri, M.; Xu, Z. S.; Wong, P. C.; Brown, R. H., Jr.; Price, D. L.; Sisodia, S. S.; Cleveland, D. W. Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. *Natl. Acad. Sci. U.S.A.* **1994**, 91, 8292-8296.
- Bruijn, L.I.; Miller, T. M.; Cleveland, D. W. Unraveling the mechanisms involved in motor neuron degeneration in ALS. *Ann. Rev. Neurosci* **2004**, 27, 723-749.
- Cao, X.; Antonyuk S. V.; Seetharaman S. V.; Whitson, L. J.; Tayloe, A. B.; Holloway, S. P.; Strange, R. W.; Doucette, P. A.; Valentine, J. S.; Tiwari, A.; Hayward, L. J.; Padua, S.; Cohlberg, J. A.; Hasnain, S. S.; Hart, P. J. Structures of the G85R variant of SOD1 in familial amyotrophic lateral sclerosis. *J. Biol. Chem.* **2008**, 283, 16169-16177.
- Cardoso, R. M. F.; Thayer, M. M.; DiDonato, M.; Lo, T. P.; Bruns, C. K.; Getzoff, E. D.; Tainer, J. A. Insight into Lou Gehrig's disease from the structure and instability of the A4V mutant of human Cu,Zn superoxide dismutase. *J. Mol. Biol.* **2002**, 324, 247-256.
- Chitin, F.; Calamal, M.; Taddei, N.; Stefani, M.; Ramponi, G.; Dobson, C. Studies of the aggregation of mutant proteins *in vitro* provide insights into the genetics of amyloid diseases. *Proc. Natl. Acad. U.S.A.* **2002**, 99, 16419-16426.

- Cleveland, D. W.; Liu, J. Oxidation versus aggregation –how do SOD1 mutants cause ALS? *Nature Med.* **2000**, 6, 1320-1321.
- Cleveland, D. W.; Rothstein, J. D. From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat. Rev. Neurosci.* **2001**, 2, 806-819.
- Cozzolino, M.; Amori, I.; Pesaresi, M. G.; Ferri, A.; Nencini, M.; Carri, M. T. Cysteine 111 affects aggregation and cytotoxicity of mutant Cu,Zn-superoxide dismutase associated with familial amyotrophic lateral sclerosis. *J. Biol. Chem.* **2008**, 283, 866-874.
- Cox, J. A.; Hensley, P. M.; Loch, C. L. Evaluation of polycation-stabilized lactate oxidase in a silica sol-gel as a biosensor platform. *Mikrochim. Acta* **2003**, 142, 1-5.
- Cudd, A.; Fridovich, I. Electrostatic interactions in the reaction mechanism of bovine erythrocyte superoxide dismutase. *J. Biol. Chem.* **1982**, 257, 11443-11447.
- Deng, H.; Shi, Y.; Furukawa, Y.; Zhai, H.; Fu, R.; Liu, E.; Gorrie, G. H.; Khan, M. S.; Hung, W.; Bigio, E. H.; Lukas, T.; Dal Canto, M. C.; O'Halloran T. V.; Siddique, T. Conversion to the amyotrophic lateral sclerosis phenotype is associated with intermolecular linked insoluble aggregates of SOD1 in mitochondria. *Proc. Natl. Acad. U.S.A.* **2006**, 103, 7142-7147.
- DiDonato, M.; Craig, I.; Huff, M. E.; Thayer, M. M.; Cardoso, R. M. F.; Kassmann, C. J.; Lo, T. P.; Bruns, C. K.; Powers, E. T.; Kelly, J. W.; Getzoff, E. D.; Tainer, J. A. ALS mutants of human superoxide dismutase form fibrous aggregates *via* framework destabilization. *J. Mol. Biol.* **2003**, 332, 601-615.
- Di Noto, L.; Whitson, L. J.; Cao, X.; Hart, P. J.; Levine, R. L. Proteasomal degradation of mutant superoxide dismutases linked to amyotrophic lateral sclerosis. *J. Biol. Chem.* **2005**, 280, 39907-39913.
- Dobson, C. M. Protein misfolding, evolution and disease. *Trends Biochem. Sci.* **1999**, 24, 329-332.
- Eggers, D. K.; Valentine J. S. Molecular confinement influences protein structure and enhances thermal protein stability. *Protein Sci.* **2001a**, 10, 250-261.
- Eggers, D. K.; Valentine J. S. Crowding and hydration effects on protein conformation: a study with sol-gel encapsulated proteins. *J. Mol. Biol.* **2001b**, 314, 911-922.
- Elam, J. S.; Taylor, A. B.; Strange, R.; Antonyuk, S.; Doucette, A. P.; Rodriguez, j. A.; Hasnain, S. S.; Hayward, L. J.; Valentine, J. S.; Yeates, T. O.; Hart, P. J. Amyloid-like filaments and water-filled nanotubes formed by SOD1 mutant proteins linked to familial ALS. *Nature* **2003**, 10, 461-467.
- Ellerby, L. M.; Nishida, C. R.; Nishida, F.; Yamanaka, S. A.; Dunn, B.; Valentine, J. S.; Zink, J. I. Encapsulation of proteins in transparent porous silicate glasses prepared by the sol-gel method. *Science* **1992**, 255, 113-1115.

- Ellis, R. J. Macromolecular crowding: An important but neglected aspect of the intracellular environment. *Curr. Opin. Struct. Biol.* **2001**, 11, 114–119.
- Forman, H. J. and Fridovich, I. On the stability of bovine superoxide dismutase. The effects of metals. *J. Biol. Chem.* **1973**, 248, 2645-2649.
- Foti, D.; Lo Curto, B.; Cuzzocrea, G.; Stroppolo, M. E.; Polizio, F.; Venanzi, M.; Desideri, A. Spectroscopic characterization of recombinant Cu,Zn superoxide dismutase from *Photobacterium leiognathi* expressed in *Escherichia coli*: evidence for a novel catalytic copper binding site. *Biochemistry* **1997**, 36, 7109-7113.
- Fridovich, I. Superoxide Dismutases. *Ann. Rev. Biochem.* **1975**, 44, 147-159.
- Fridovich, I. Superoxide radical and superoxide dismutases. *Ann. Rev. Biochem.* **1995**, 64, 97-112.
- Fujiwara, N.; Miyamota, Y.; Ogasahara, K.; Takahashi, M.; Ikegami, T.; Takamiya, R.; Suzuki K.; Tanigushi, N. Different immunoreactivity against monoclonal antibodies between wild-type and mutant copper/zinc superoxide dismutase linked to amyotrophic lateral sclerosis. *J. Biol. Chem.* **2005**, 280, 5061-5070.
- Furukawa, Y.; Torres, A. S.; O'Halloran, T. V. Oxygen-induced maturation of SOD1: A key role for disulfide formation by the copper chaperone CCS. *EMBO J.*, **2004**, 23, 2872-2881.
- Goto, J. J.; Zhu, H.; Sanchez, R. J.; Nersissian, A.; Gralla, E. B.; Valentine, J. S. Loss of *in vitro* metal ion binding specificity in mutant copper-zinc superoxide dismutases associated with familial amyotrophic lateral sclerosis. *J. Biol. Chem.* **2000**, 275, 1007-1014.
- Gurney, M.; Pu, H.; Chiu, A. Y.; Canto, M. C. D.; Polchow, C. Y.; Alexander, D. D.; Caliendo, J.; Hentati, A.; Kwon, Y. W.; Deng, H. X.; Chen, W.; Zhai, P.; Sufit, R. L.; Siddique, T. Motor neuron degeneration in Mice that express a human Cu,Zn superoxide dismutase mutation. *Science* **1994**, 264, 1772-1775.
- Hall, P. G.; Pidduck, A. J.; Wright, C. J. Inelastic neutron scattering by water adsorbed on silica. *J. Colloid Interface Sci.* **1981**, 79, 339.
- Hammes, G. G. *Spectroscopy for the biological sciences*; Wiley & Sons: New Jersey, 2005; Chapter 4.
- Hirano, A.; Donnenfeld, H.; Sasaki, S.; Nakamo, I. Fine structural observations of neurofilamentous changes in amyotrophic lateral sclerosis. *J. Neuropathol. Exp. Neurol.* **1984**, 43, 461-470.
- Hornemann, S; Glockshuber, R. A scrapie like unfolding intermediate of the prion protein domain PrP (121-231) induced by acidic pH. *Proc. Natl. Acad. U.S.A.* **1998**, 95, 6010-6014.
- <http://www.als.org>
- <http://www.hamptonresearch.com>

<http://www.scripps.edu/cgi-bin/cdputnam/protcalc3>

- Hoang, G. C.; Lee, H. S. Pore size control of silica gels in basic water conditions using sol-gel processing. *Proceedings of the 5th international conference on properties and applications of dielectric materials*, Seoul, Korea, May 25-30, 1997.
- Hough, M. A.; Grossmann, J. G.; Antonyuk, S. V.; Strange, R. W.; Doucette, P. A.; Rodriguez, J. A.; Whitson, L. J.; Hart, P. J.; Hayward, L. J.; Valentine, J. S.; Hasnain S. S. Dimer destabilization in superoxide dismutase may result in disease-causing properties: Structures of motor neuron disease mutants. *Proc. Natl. Acad. U.S.A.* **2004**, 101, 5976-5981.
- Hwang, C.; Sinskey, A. J. Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* **1992**, 257, 1496-1502.
- Jain, S.; Ahluwalia, J.C. Different scanning calorimetric studies on the effect of ammonium and tetraalkylammonium halides on the stability of lysozyme. *Biophys. Chem.* **1996**, 59, 171-177.
- Jin, W.; Brennan, J. D. Properties and applications of proteins encapsulated within sol-gel derived materials. *Anal. Chim. Acta* **2002**, 461, 1-36.
- Johnston, J. A.; Dalton, M. J.; Gurney, M. E.; Kopito, R. R. Formation of high molecular weight complexes of mutant Cu,Zn-superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis. *Proc. Natl. Acad. U.S.A.* **2000**, 97, 12571-12576.
- Julien, J. P. Amyotrophic lateral sclerosis: unfolding the toxicity of the misfolded. *Cell* **2001**, 104, 581-591.
- Kaytor, M. D.; Warren, S. T. Aberrant protein deposition, and neurological disease. *J. Biol. Chem.* **1999**, 274, 37507-37510.
- Kentsis, A.; Sosnick T. R. Trifluoroethanol promotes helix formation by destabilizing backbone exposure: Desolvation rather than native hydrogen bonding defines the kinetic pathway of dimeric coiled coil folding. *Biochemistry* **1998**, 37, 14613-14622.
- Khan, I.; Shannon, C. F.; Dantsker, D.; Friedman, A. J.; Perez-Gonzalez-de-Apodaca, J.; Friedman, J. M. Sol-gel trapping of functional intermediates of hemoglobin: Geminate and biomolecular recombination studies. *Biochemistry* **2000**, 39, 16099-16109.
- Khare, S. D.; Caplow, M.; Dokholyan, N. V. The rate and equilibrium constants for a multistep reaction sequence for the aggregation of superoxide dismutase in amyotrophic lateral sclerosis. *Proc. Natl. Acad. U.S.A.* **2004**, 101, 15094-15099.
- Klung-Roth, D.; Fridovich, I.; Rabani, J. Pulse radiolytic investigation of superoxide catalyzed disproportionation. Mechanism for bovine superoxide dismutase. *J. Am. Chem. Soc.* **1973**, 95, 2786-2790.

- Koga, Y.; Katayamagi, H.; Davies, J. V.; Kato, H.; Nishikawa, K.; Westh, P. The effects of chloride salts of some cations on the molecular organization of H₂O towards understanding the Hofmeister series II. *Bull. Chem. Soc. Jpn.* **2006**, 79, 1347-1354.
- Li, T. M.; Alberman, E.; Swash, M. Comparison of sporadic and familial disease amongst 580 cases of motor neuron disease. *J. Neurol. Neurosurg. Psychiatry*, **1988**, 51, 778-784.
- Lindberg, M. J.; Tibell, L.; Oliveberg, M. Common denominator of Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis: decreased stability of the apo state. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, 99, 16607-16612.
- Liu, D.; Zhang, Y.; Chen, C. - C.; Mou, C. - Y.; Poole, P. H.; Chen, S. H. Observation of the density minimum in deeply supercooled confined water. *Proc. Natl. Acad. U.S.A.* **2007**, 104, 9570-9574.
- Lynch, S. M.; Boswell, S. A.; Colón, W. Kinetic stability of Cu/Zn superoxide is dependent on its metal ligands: Implications for ALS. *Biochemistry* **2004**, 43, 16525-16531.
- Marklund, S. L.; Andersen, P. M.; Forsgren, L.; Nilsson, P.; Ohlsson, P. I.; Wikander, G.; Oberg, A. Normal binding and reactivity of copper in mutant superoxide dismutase isolated from amyotrophic lateral sclerosis patients. *J. Neurochem.* **1997**, 69, 675-681.
- Meiering, E. M. The threat of instability: Neurodegeneration predicted by protein destabilization and aggregation propensity. *PLoS Biol* [Online] **2008**, 6, e193.doi:10.137/journal.pbio.0060193.
- Minton, A. P. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J. Biol. Chem.* **2001**, 276, 10577-10580.
- Nguyen, D. T.; Smit, M.; Dunn, B.; Zink, J. I. Stabilization of creatine kinase encapsulated in silicate sol-gel materials and unusual temperature effects on its activity. *Chem. Mater.* **2002**, 14, 4300-4306.
- Parge, H. E.; Hallewell, R. A.; Tainer, J. A. Atomic structures of wild-type and thermostable mutant recombinant human Cu,Zn superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, 89, 6109-6113.
- Pierre, A. C. The Sol-Gel Encapsulation of Enzymes. *Biocatal. Biotrans.* **2004**, 22, 145-170.
- Potter, S. Z.; Zhu, H.; Shaw, B. F.; Rodriguez, G.; Doucette, P.; Sohn, S. H.; Durazo, A.; Faull, K. F.; Gralla, E. B.; Nersissian, A. M.; Valentine, J. S. Binding of a single ion to one subunit of copper-zinc superoxide dismutase apoprotein substantially influences the structure and stability of the entire homodimeric protein. *J. Am. Chem. Soc.* **2007**, 129, 4575-4583.

- Rakhit, R.; Crow J. P.; Lepock J. R.; Kondejewski, L. H.; Cashman N. R.; Chakrabartty, A. Monomeric Cu,Zn-superoxide dismutase is a common misfolding intermediate in the oxidation models of sporadic and familial lateral sclerosis. *J. Biol. Chem.* **2004**, 279, 15499-15504.
- Rakhit, R.; Cunningham, P.; Furtos-Matei, A.; Dahan, S.; Qi, X-F.; Crow, J. P.; Cashman N. R.; Kondejewski L. H.; Chakrabartty, A. Oxidation-induced misfolding and aggregation of superoxide dismutase and its implications for amyotrophic lateral sclerosis. *J. Biol. Chem.* **2002**, 277, 47551-47556.
- Ray, S. S.; Nowak, R. J.; Strokovich, K.; Brown, R. H., Jr.; Walz, T.; Lansbury, P. T., Jr. An intersubunit disulfide bond prevented in vitro aggregation of a superoxide dismutase-1 mutant linked to familial amyotrophic lateral sclerosis. *Biochemistry* **2004**, 43, 4899-4905.
- Reaume, A. G.; Elliot, J. L.; Hoffman, E. K.; Kowall, N. W.; Ferrante, R. J.; Siwek, D. R.; Wilcox, H. M.; Flood, D. G.; Beal, M. F.; Brown, R. H., Jr.; Scott, R. W.; Snider, W. D. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat. Genet.* **1996**, 13, 43-47.
- Rocha, V.; Eggers, D. K. Hydrophobic, organically-modified silica gels enhance the secondary structure of encapsulated apomyoglobin. *Chem. Commun.* **2007**, 12, 1266-1268.
- Rodriguez, J. A.; Shaw, B. F.; Durazo, A.; Sohn, S.H.; Doucette, P. A.; Nersissian, A. M.; Faull, K. F.; Eggers, D. K.; Tiwari, A.; Hayward, L. J.; Valentine, J. S. Destabilization of apoprotein is insufficient to explain Cu,Zn-superoxide dismutase-linked ALS pathogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, 102, 10516-10521.
- Rodriguez, J. A.; Valentine, J. S.; Eggers, D. K.; Roe, J. A.; Tiwari, A.; Brown, R. H. jr.; Hayward, L. J. Familial amyotrophic lateral sclerosis-associated mutations decrease the thermal stability of distinctly metallated species of human copper/zinc superoxide dismutase. *J. Biol. Chem.* **2002**, 277, 15932-15937.
- Roe, J. A.; Butler A.; Scholler D. M.; Valentine J. S. Differential scanning calorimetry of Cu,Zn-superoxide dismutase, the apoprotein, and its zinc-substituted derivatives. *Biochemistry* **1998**, 27, 950-958.
- Roe, J. A.; Wiedau-Pazos, M.; Moy, V. N.; Goto, J. J.; Gralla, E. B.; Valentine, J. S. In vivo peroxidative activity of FALS-mutant human CuZnSODs expressed in yeast. *Free Radical Biol. Med.* **2002**, 32, 169- 174.
- Rosen, D. R.; Siddique, T.; Patterson, D.; Figlewicz, D. A.; Sapp, P.; Hentati, A.; Donaldson, D.; Goto, J.; O'Regan, J. P.; Deng, H. X. et al., Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **1993**, 362, 59-62.

- Shaw, B. F. and Valentine, J. S. How do ALS-associated mutations in superoxide dismutase 1 promote aggregation of the protein? *Trends Biochem. Sci.* **2006**, 32, 78-85.
- Shaw, B. F.; Durazo, A.; Nersissian, A. M.; Whitelegge, J. P. Faull, K. F.; Valentine, J. S. Local unfolding in a destabilized, pathogenic variant of superoxide dismutase 1 observed with H/D exchange and mass spectrometry. *J. Biol. Chem.* **2006**, 281, 18167-18176.
- Sreerama, N.; Venyaminov, S. Y.; Woody, R. W. Estimation of the number of α -helical and β -strand segments in proteins using circular dichroism spectroscopy. *Protein Sci.* **1999**, 8, 320-380.
- Sreerama, N.; Woody, R. W. Computation and analysis of protein circular dichroism spectra. *Methods. Enzymol.*, **2004**, 38, 318-351.
- Sreerama, N.; Woody, R. W. Estimation of protein secondary structure from CD spectra: Comparison of CONTIN, SELCON and CDSSTR methods with an expanded reference set. *Anal. Biochem.* **2000**, 282, 252-260.
- Srisailam, S.; Kumar, T. K. S.; Rajalingam, D.; Kathir, K. M.; Sheu, H-S.; Jan, F-J.; Chao, P-C. Amyloid-like fibril formation in an all β -barrel protein. *J. Biol. Chem.* **2003**, 278, 17701-17709.
- Stanley, H. E.; Kumar, P.; Xu, L.; Yan, Z.; Mazza, M. G.; Buldyrev, S. V.; Chen, S.-H.; Mallamace, R. New results on water in bulk, nanoconfined, and biological environments. *AIP Conf. Proc.* **2007**, 965, 193-212.
- Stathopoulos, P. B.; Rumfeldt, J. A. O.; Scholz, G. A.; Irani, R. A.; Frey, H. E.; Hallewell, R. A.; Lepock, J. R.; Meiering, E. M. Cu / Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis show enhanced formation of aggregates in vitro. *Proc. Natl. Acad. SCI. U.S.A.* **2003**, 100, 7012-7026.
- Subramaniam, J. R.; Lyons, W. E.; Liu, J.; Bartnikas, T. B.; Rothstein, J.; Price, D. L.; Cleveland, D. W.; Gitlin, J. D.; Wong, P. C. Mutant SOD1 causes motor neuron disease independent of copper chaperone-mediated copper loading. *Nat. Neurosci.* **2002**, 5, 301-307.
- Symons, M. C. R. Water structure and reactivity. *Acc. Chem. Res.* **1981**, 14, 179-187.
- Takamuku, T.; Yamagami, M.; Wakita, H.; Masuda, Y.; Yamaguchi, T. Thermal property, structure, and dynamics of supercooled water in porous silica by calorimetry, neutron scattering, and NMR relaxation. *J. Phys. Chem. B* **1997**, 101, 5730-5739.
- Tiwari, A.; Hayward, L. J. Familial amyotrophic lateral sclerosis mutants of copper/zinc superoxide dismutase are susceptible to disulfide reduction. *J. Biol. Chem.* **2003**, 278, 5984-5992.

- Valentine, J. S. Do oxidatively modified proteins cause ALS? *Free Radical Biol. Med.* **2002**, 33, 1314-1320.
- Valentine, J. S.; Doucette, P. A.; Potter, S. Z. Copper-zinc superoxide dismutase and amyotrophic lateral sclerosis. *Annu. Rev. Biochem.* **2005**, 74, 563-593.
- Valentine, J. S.; Hart P. J. Misfolded CuZnSOD and amyotrophic lateral sclerosis. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, 100, 3617-3622.
- Valentine, J. S.; Pantoliano, M. W.; McDonnell, P. J.; Burger, A. R.; Lippard, S. J. pH-dependent migration of copper (II) to the vacant zinc-binding site of zinc-free bovine erythrocyte superoxide dismutase. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, 76, 4245-4249.
- van den Berg, B.; Ellis, J.; Dobson, C. M. Effects of macromolecular crowding on protein folding and aggregation. *EMBO J.* **1999**, 18, 6927-6933.
- Voet, D.; Voet, J. D. *Biochemistry*, 3rd ed.; Wiley & Sons: New Jersey, 2004.
- von Hippel, P. H.; Wong, K.-Y. On the conformational stability of globular proteins: The effects of various electrolytes and non-electrolytes on the thermal ribonuclease transition. *J. Biol. Chem.* **1965**, 240, 3909-3923.
- Wang, J.; Xu, G.; Borchelt, D. R. Mapping superoxide dismutase 1 domains of non-native interaction: Roles of intra- and intermolecular disulfide bonding in aggregation. *J. Neurochem.* **2006**, 96, 1277-1288.
- Wang, J. M.; Takeda, A.; Yang, J. T.; Wu, C. Conformation of concanavalin A and its fragments in aqueous solution and organic solvent-water mixtures. *J. Protein Chem.* **1992**, 11, 157-164.
- Wang, Q.; Johnson, J. L.; Agar, N. Y. R.; Agar, J. N. Protein aggregation and protein instability govern familial amyotrophic lateral sclerosis patient survival. *Plos Biol* [Online] **2008**, 6, e170. doi:10.1371/journal.pbio.0060170.
- Watanabe, M.; Dykes-Hoberg, M.; Culotta, V. C.; Price, D. L.; Wong, P. C.; Rothstein, J. D. Histological evidence of protein aggregation in mutant SOD1 transgenic mice and in amyotrophic lateral sclerosis neural tissues. *Neurobiol. Dis.* **2001**, 8, 933-941.
- Wei, Y.; Dong, H.; Xu, J.; Feng, Q. Simultaneous immobilization of horseradish peroxidase and glucose oxidase in mesoporous sol-gel host materials. *Chem PhysChem* **2003**, 3, 802-808.
- Wiedau-Pazos, M.; Goto, J. J.; Rabizadeh, S.; Gralla, E. B.; Roe, J. A.; Lee, M. K.; Valentine, J. S.; Bredesen, D. E. Altered reactivity of superoxide dismutase in familial amyotrophic lateral sclerosis. *Science* **1996**, 271, 515-518.
- Wright, J. D.; Sommerdijk, N. A. J. M. *Sol-Gel Materials Chemistry and Applications*; CRC Press: Boca Raton, FL, **2001**; pp 15-22.