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Abstract
Amyotrophic lateral sclerosis is a devastating neurodegenerative disease. The mechanism that underlies amyotrophic lateral sclerosis (ALS) pathology remains unclear, but protein inclusions are associated with all forms of the disease. Apart from pathogenic proteins, such as TDP-43 and SOD1, other proteins are associated with ALS inclusions including small heat shock proteins. However, whether small heat shock proteins have a direct effect on SOD1 aggregation remains unknown. In this study, we have examined the ability of small heat shock proteins αB-crystallin and Hsp27 to inhibit the aggregation of SOD1 in vitro. We show that these chaperone proteins suppress the increase in thioflavin T fluorescence associated with SOD1 aggregation, primarily through inhibiting aggregate growth, not the lag phase in which nuclei are formed. αB-crystallin forms high molecular mass complexes with SOD1 and binds directly to SOD1 aggregates. Our data are consistent with an overload of proteostasis systems being associated with pathology in ALS.

Keywords
sod1, suppress, hsp27, crystallin, b, vitro, proteins, aggregation, shock, heat, small, CMMB

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The small heat shock proteins αB-crystallin and Hsp27 suppress SOD1 aggregation in vitro

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Key words: Amyotrophic Lateral Sclerosis, SOD1, protein aggregation, small heat shock proteins, molecular chaperones, αB-crystallin

Abstract (150 words)

Amyotrophic lateral sclerosis is a devastating neurodegenerative disease. The mechanism that underlies ALS pathology remains unclear, but protein inclusions are associated with all forms of the disease. Apart from pathogenic proteins, such as TDP-43 and SOD1, other proteins are associated with ALS inclusions including small heat shock proteins. However, whether small heat shock proteins have a direct effect on SOD1 aggregation remains unknown. In this study we have examined the ability of small heat shock proteins αB-crystallin and Hsp27 to inhibit the aggregation of SOD1 *in vitro*. We show that these chaperone proteins suppress the increase in thioflavin T fluorescence associated with SOD1 aggregation primarily through inhibiting aggregate growth, not the lag phase in which nuclei are formed. αB-crystallin forms high molecular mass complexes with SOD1 and binds directly to SOD1 aggregates. Our data are consistent with an overload of proteostasis systems being associated with pathology in ALS.

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that is characterised by the progressive and selective death of upper and lower motor neurons, leading to loss of muscle control, muscle atrophy and invariably death, generally within 3-5 years of diagnosis. Neurodegeneration in ALS has been attributed to a variety of processes including glutamate excitotoxicity, oxidative stress, disruption of neurofilaments and axonal transport, protein aggregation, mitochondrial dysfunction, endoplasmic reticulum stress, and (most recently) dysfunctional RNA metabolism (Pasinelli & Brown 2006). Although the cause of sporadic forms of ALS remains a mystery, there is a rapidly growing list of genes in which mutations cause familial ALS (fALS; accounting for 5-10% of all ALS cases). These include SOD1 (Rosen et al. 1993), alsin (Yang et al. 2001), senataxin (Chen et al. 2004), FUS/TLS (Kwiatkowski et al. 2009; Vance et al. 2009), VAPB (Nishimura et al. 2004), TDP-43 (Kabashi et al. 2008; Sreedharan et al. 2008), optineurin (Maruyama et al. 2010), VCP (Johnson et al. 2010) and c9orf72 (DeJesus-Hernandez et al. 2011). In common with other neurodegenerative diseases, such as Alzheimer's disease, Creutzfeldt-Jakob disease, Parkinson's disease, and Huntington's disease, there is growing evidence that protein aggregates are closely associated with degeneration in all forms of ALS (Leigh et al. 1991; Ticozzi et al. 2010). Indeed, there are some that consider ALS a proteinopathy (Strong et al. 2005).

The best-studied fALS cases are from families possessing mutations in the gene encoding copper/zinc superoxide dismutase (Cu/Zn SOD, SOD1). There are over 140 different mutations dispersed over the whole sequence of the SOD1 gene that can cause ALS. What these mutations have in common is the ability to destabilise the structure of the protein, shortening its half-life (Borchelt et al. 1994) and increasing its propensity to aggregate into oligomeric forms (Banci et al. 2007) and form insoluble material in cells (Prudencio et al. 2009). Predictions using the Chiti-Dobson equation (that predicts aggregation propensity) have suggested that protein instability and aggregation propensity is a risk factor for SOD1 associated fALS (Wang et al. 2008b). It is thought that the mutant versions of SOD1 never realise their proper native structure, and therefore can cause cellular malfunction if they are not rapidly removed (Hart 2006). *In vitro* SOD1 follows a two phase kinetic model of aggregation that is common to most amyloid forming proteins, which includes a (rate-limiting) lag phase in which oligomeric nuclei are formed, followed by a rapid growth phase

and subsequent plateau phase in which aggregate growth has reached an equilibrium (Chattopadhyay et al. 2008).

Although mutant SOD1 is aggregation prone, it is maintained in a soluble form in young mice (Wang et al. 2009). This is presumably due to the efficient network of processes in place in cells to maintain protein homeostasis (proteostasis). It is thought that with aging these proteostasis systems can become overloaded or defective, resulting in persistent deposits of aggregated protein associated with disease pathology (Ben-Zvi et al. 2009). Aggregates of SOD1 are found in motor neurones and astrocytes in fALS patients and mutant SOD1 expressing mice (Bruijn et al. 1998). The accumulation of this misfolded protein signifies a breakdown in the quality control system that prevents protein aggregation or an inability of these systems to cope with the increased load brought about by mutant SOD1 expression in ALS (Wang et al 2009). There are many other proteins associated with SOD1 deposits in vivo including molecular chaperones HSP70 and αB-crystallin, and structural proteins such as vimentin, neurofilament heavy chain and tubulin (Bergemalm et al. 2010). The reason why these proteins are present in these deposits is unknown, however, it has been proposed that, at least in the case of chaperones, it may signify their failed attempt to keep the aggregating protein soluble in solution (Muchowski & Wacker 2005).

The small heat shock proteins (sHsps) αB-crystallin and Hsp27 are of particular interest because they co-localise with astrocytic inclusions in humans (Kato et al. 1997), and both Hsp25 (the mouse ortholog of human Hsp27) and αB-crystallin increase in abundance in the spinal cord as ALS progresses in mouse models (Vleminckx et al. 2002; Wang et al. 2008a; Wang et al. 2005). αB-crystallin and Hsp25 have also been identified as components of inclusions from mutant SOD1 (G127X, G93A, D90A and G37R) mice using proteomics techniques on isolated inclusions (Bergemalm et al 2010) and detergent insoluble fractions (Basso et al. 2009). Moreover, SOD1 can be immunoprecipitated using αB-crystallin antibodies in cell models of SOD1 associated ALS (Shinder et al. 2001). In HEK293 cells over-expression of αB-crystallin has been shown to suppress mutant SOD1 aggregation (Karch & Borchelt 2010), and αB-crystallin has been shown to inhibit the movement of SOD1 into the insoluble fraction of tissue homogenates from mutant SOD1 mice when incubated at 37^{o}C (Wang et al 2005). Hsp27 has been shown to protect cultured neurons from SOD1 proteotoxicity (Patel et al. 2005) but this conflicts with work in cell culture models that reports

that Hsp27 over-expression does not protect Neuro2a cells from SOD1-associated toxicity (Krishnan et al. 2006). In animal models, knock-down of αB-crystallin does not affect the amount of insoluble SOD1 nor does it significantly alter lifespan of transgenic mice (Karch & Borchelt 2010). Similarly, while over-expression of Hsp27 slows the early phase of disease it does not alter lifespan of SOD1 mice (Sharp et al. 2008). This may be, at least in part, due to the fact that robust expression of αB-crystallin and Hsp25 is largely restricted to glial cells. However, there have been no studies that have investigated whether αB-crystallin or Hsp27 directly interact and suppress the aggregation of SOD1 outside of the complex cellular milieu. The aim of this work was therefore to examine the ability and mechanism by which the sHsps αB-crystallin and Hsp27 suppress SOD1 aggregation *in vitro*.

Results and Discussion

Previous work has demonstrated that SOD1 is aggregation prone in its apo and disulphide reduced state (Chattopadhyay et al 2008; Furukawa et al. 2008). Moreover, a large proportion of human mutant SOD1 is found to exist in a demetallated and reduced form in transgenic mouse models (Jonsson et al. 2006). In the current study we have used DTT and EDTA to reduce the disulphide bonds and remove bound metal ions to promote aggregation of SOD1. We observed that, under the conditions used in this study, there was no increase in ThT fluorescence associated with WT SOD1 until high concentrations of DTT (50 mM) were used (Figure 1A). In the presence of 50 mM DTT, WT SOD1 aggregation was defined by a lag phase of ~5 h and maximum ThT fluorescence was reached after ~10 h of incubation. Similarly, G93A SOD1 aggregation was dependent on the presence of DTT, however, lower concentrations (10 mM) were required to initiate aggregation (Figure 1B). This is consistent with work that shows that native state of G93A SOD1 is more destabilised than WT SOD1 (Svensson et al. 2010). However, when high concentrations of DTT were used (50 mM), the rate of aggregation of G93A SOD1 was very similar to WT SOD1 under the same conditions. This indicates that while the SOD1 mutations destabilise the native conformation, when WT SOD1 is destabilised by extreme conditions it will aggregate at a similar rate. These data are consistent with computational (Wang et al 2008b) and cell culture models (Prudencio et al 2009), that indicate a correlation between aggregation propensity of SOD1 and disease severity..

Previous studies have reported that although over-expression of αB-crystallin can reduce insoluble aggregate formation in cell models of SOD1 aggregation, knock-down of αB-crystallin in a G93A SOD1 mouse model of ALS does not affect the total amount of insoluble protein measured (Karch & Borchelt 2010). As a result, it was not clear if the suppression of aggregation in cell models was due to a direct role in halting aggregation or whether boosting the capacity of the cell's proteostasis machinery was enough to quell aggregation. In order to test whether sHsps could indeed directly suppress SOD1 aggregation we used our *in vitro* aggregation assay and recombinant forms of αB-crystallin and Hsp27. Co-incubation of G93A SOD1 with αB-crystallin resulted in a significant decrease in ThT fluorescence associated with SOD1 aggregation (Figure 2A) but it did not alter the lag phase (which remained at ~ 5 h in the presence or absence of αB-crystallin). These results are consistent with αB-crystallin having a direct role in suppressing SOD1 aggregation in cell models over-expressing αB-crystallin. The lack of an obvious change in phenotype upon knockout of αB-crystallin in the G93A SOD1 mouse model of ALS may be attributable to the overlap in chaperone function of the small heat shock proteins, i.e. other members of the sHsp family may have compensated for the loss of αB-crystallin in these mice. Alternatively, αB-crystallin may not be expressed at sufficient levels in neurons where SOD1 aggregation occurs in these mice. In support of this, it is well documented that αB-crystallin expression in motor neurones is low in comparison to surrounding glia (Vleminckx et al 2002) and the initial stages of SOD1 aggregation occurs in motor neurones in mice (Stieber et al. 2000).

We next tested whether the Hsp27 is also able to inhibit G93A SOD1 aggregation *in vitro*. As seen for αB-crystallin, co-incubation of G93A SOD1 with Hsp27 resulted in a decrease in ThT fluorescence associated with SOD1 aggregation (Figure 2B). Again, the presence of Hsp27 did not significantly alter the lag phase. However, at a molar ratio of 1:0.01 (SOD1:chaperone) Hsp27 was significantly less effective at inhibiting G93A SOD1 aggregation when compared to αB-crystallin (αB-crystallin 78 ± 5% compared to Hsp27 54 ± 6%, $p<0.05$). This data indicates that the ability of Hsp27 to suppress SOD1 proteotoxicity in cultured neurons (Patel et al 2005) is likely to be attributable to the chaperone activity of Hsp27. This is consistent with increased survival of motor neurons in early stage disease in Hsp27 over-expressing SOD1 mice (Sharp et al 2008). The lack of an effect on longevity in this model is most likely attributable to an overwhelming of the chaperone: as disease progressed Hsp27 levels in motor neurons decreased (Sharp et al 2008).

Previous cell culture experiments have detected the formation of stable complexes between G93A SOD1 aggregates and αB-crystallin, however, it was unclear whether this interaction was a direct one or mediated through another (unidentified) factor (Shinder et al 2001). The binding of sHsps to SOD1 aggregates may account, at least in part, for the depleted levels of Hsp25 reported in the cytosolic fractions of motor neurons in G93A SOD1 mice (Maatkamp et al. 2004). Stable complexes between SOD1 and sHsps may also explain the presence of Hsp25 and αB-crystallin in the insoluble fractions of spinal cord extracts from transgenic mice with ALS symptoms (Wang et al 2008a; Wang et al 2005). Therefore, to test if αB-crystallin is capable of binding directly to SOD1 and forming a stable complex with it, we incubated WT SOD1 with 50 mM DTT in the absence or presence of αB-crystallin. Aggregation of WT SOD1 when incubated alone was more rapid in this assay than seen previously (compare the green line in Figure 1A with the blue line in Figure 3A) due to the increased rate of shaking used for this assay, i.e. 300 revolutions per minute (rpm) versus 120 rpm. The rate of agitation is known to play a significant role in the kinetics of aggregation due to it promoting nuclei formation and therefore secondary nucleation (Knowles et al 2009; Xue et al 2008). Addition of αB-crystallin resulted in a concentration-dependent decrease in ThT fluorescence associated with WT SOD1 aggregation, such that, a molar ratio of 1:1 (SOD1: αB-crystallin), there was nearly complete inhibition (Figure 3A). Size exclusion chromatography and SDS-PAGE of the fractions eluting from the column demonstrated that, prior to incubation, SOD1 elutes as a single peak at an elution volume of 15.8 mL, consistent with its dimeric form in the native state (Figure 3B). αB-crystallin primarily elutes as a broad peak centred at an elution volume of 11.1 mL due to its polydisperse oligomeric state (Haley et al 1998). The sample containing both WT SOD1 and αB-crystallin (at a 1:1 molar ratio) eluted primarily as a very broad peak centred at an elution volume of 10.8 mL. This peak contains both SOD1 and αB-crystallin. Moreover, there was a decrease in intensity of the dimeric SOD1 peak (i.e. at an elution volume of 15.8 mL). These data are consistent with the formation of a high molecular mass complex between SOD1 and αB-crystallin. The formation of a high molecular mass complex between αB-crystallin and aggregating proteins reflects the well-described ‘holdase’ type chaperone mechanism of sHsps and is thought to enable a maintenance of aggregating protein in solution (Ecroyd & Carver 2009).

Taken together, our observations are consistent with the sHsps inhibiting SOD1 aggregation through an effect upon the growth phase of the aggregation kinetics rather than upon the lag phase. That is, while both αB-crystallin and Hsp27 significantly reduced the extent of SOD1 aggregation, the lag phase of the reaction was not significantly changed in the presence or absence of the chaperones (see Figure 2 and Figure 3A). If a two-phase kinetic model is used to model the aggregation process of SOD1, these data imply that both Hsp27 and αB-crystallin inhibit SOD1 aggregation by acting primarily upon fibril elongation rather than the formation of fibril nuclei. Thus, our data suggests that sHsps bind to larger oligomeric species formed during SOD1 aggregation rather than partially folded monomers and pre-nuclei oligomers. Such a mechanism is consistent with recent work in which αB-crystallin has been reported to be capable of binding to preformed fibrils and, in doing so, suppress further fibril formation (Shammas et al. 2011; Waudby et al. 2010). Moreover, in both humans and mouse models of ALS, αB-crystallin is primarily associated with the insoluble inclusions (Basso et al 2009; Bergemalm et al 2010; Kato et al 1997). Thus, we directly tested whether αB-crystallin is capable of binding to mature SOD1 aggregates using a dot blot assay (Figure 3C). After centrifugation and washing, a proportion of aggregated SOD1 could be detected in the pellet (P). In contrast, when aggregated WT SOD1 and αB-crystallin were incubated together αB-crystallin was also detected in the pellet (P) fraction, consistent with it binding to aggregated SOD1. There was no apparent difference in the amount of SOD1 in the pellet fraction when αB-crystallin was incubated with this aggregated sample. The ability of αB-crystallin to bind to aggregated forms of SOD1 may, at least in part, explain the presence of αB-crystallin in SOD1 positive inclusions in ALS patients and its association with inclusions in mouse models.

In summary the *in vitro* model of SOD1 aggregation used in this study has provided evidence that the sHsps αB-crystallin and Hsp27 can directly interact with and inhibit SOD1 aggregation. These findings, along with those that show that upregulation of sHsps (and other chaperones, such as Hsp70) protect against SOD1 proteotoxicity (Gifondorwa et al. 2007; Kalmar et al. 2012; Sharp et al 2008) raises the question as to why inclusion formation still occurs *in vivo*. A possible explanation is that systems that normally act to maintain proteostasis are overloaded by the increased burden brought about by SOD1 aggregation, or the decrease in the soluble pool of chaperone proteins that occurs because of this, or both. It is important to note that the aggregation assays in this study were exclusively conducted under

reducing conditions. While disulfide bond reduction is thought to be an important step in the monomerisation and subsequent aggregation of SOD1 (Chattopadhyay et al 2008; Karch et al. 2009), several theories of SOD1 toxicity focus upon oxidative stress as an important factor in ALS pathogenesis (Pasinelli & Brown 2006). Moreover, previous studies with G93A SOD1 mice have identified heterogeneous SOD1 aggregates which varied in form with disease progression (Sasaki et al. 2005). Given that different reaction conditions (*in vitro*) have been reported to produce differing species of SOD1 aggregates (Chattopadhyay et al 2008; Rakhit et al. 2002; Stathopulos et al. 2003) it is possible that the cellular conditions and aggregation pathway also vary with disease progression. This may, at least in part, explain the contradictory reports of protection from sHsps in the literature.

While the rapid and predictable progression of SOD-related ALS confirms the ability of toxic G93A SOD1 species to evade *in vivo* proteostatic systems, the mechanism by which G93A SOD1 evades these systems remains unknown. This study was able to confirm the ability of Hsp27 and αB-crystallin to inhibit G93A SOD1 aggregation *in vitro*. Since previous studies have suggested that overexpression of chaperones is insufficient to attenuate the progression ALS in mouse models (Krishnan et al. 2008) further investigation to clarify the mechanism by which mutant SOD1 escapes the proteostatic machinery might provide clues to a possible treatment for ALS.

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Figure legends

Figure 1 – The reduction-dependent aggregation of SOD1 in vitro. Wild-type (a) or G93A (b) SOD were incubated at 30 μM in 10 mM potassium phosphate buffer containing 5 mM EDTA, pH 7.4 whilst shaking (300 rpm for 5 min each cycle, 15 min cycles) at 37ºC in the absence (red) or presence of 10 mM (blue) or 50 mM (green) DTT. The buffer alone sample is also shown (orange). The amount of ThT fluorescence (excitation at 440 nm and emission at 490nm) was monitored over time using a microplate-reader and the change in ThT fluorescence is reported as a mean ± SEM of three replicates. The data is representative of 3 independent experiments.

Figure 2 - The small heat shock proteins αB-crystallin and Hsp27 inhibit the in vitro aggregation of G93A SOD1. G93A SOD1 was incubated at 30 μM in 10 mM potassium phosphate buffer containing 5 mM EDTA, pH 7.4 whilst shaking (300 rpm for 5 min each cycle, 15 min cycles) at 37ºC in the absence or presence of (a) αB-crystallin, or (b) Hsp27. The amount of ThT fluorescence (excitation at 440 nm and emission at 490nm) was monitored over time using a microplate-reader and the change in ThT fluorescence is reported as a mean ± SEM of three replicates. The data is representative of at least 3 independent experiments. Molar ratios (SOD1: chaperone) are shown on the right.

Figure 3 –αB-crystallin inhibits the in vitro aggregation of WT SOD 1 by forming a stable complex. (a) Wild-type SOD1 was incubated at 30 μM in 10 mM potassium phosphate buffer containing 5 mM EDTA, pH 7.4 whilst shaking (120 rpm for 5 min each cycle, 15 min cycles) at 37ºC in the absence or presence of αB-crystallin and the amount of ThT fluorescence (excitation at 440 nm and emission at 490nm) was monitored over time using a microplate-reader. Molar ratios (SOD1: chaperone) are shown on the right. Inset: The percent inhibition of the increase in ThT fluorescence afforded by αB-crystallin. Results shown are mean ± SEM of three replicates. **(b)** Size exclusion chromatography and SDS-PAGE of the eluate to establish whether αB-crystallin prevents WT SOD1 aggregation by forming a complex with it. Samples containing non-incubated WT SOD1 (30 μM, blue), αB-crystallin alone (30 μM, purple) or WT SOD 1 and αB-crystallin (at a molar ratio of 1:1 and recovered at the end of the assay described in a) (orange) were applied to a Superdex 200 HR 10/30 column and eluted with PBS at a flow rate of 0.5 mL/min. Eluate from the column was

collected into 0.5 mL fractions. The sample loaded onto the column (L) (diluted five-fold with PBS) and every second fraction eluting between 8 to 17 mL were subjected to SDS-PAGE analysis. (c) Immuno-dot blot used to detect the interaction of αB-crystallin with aggregated SOD1. Aggregated WT SOD1 (20 μM) was incubated in the absence or presence of αB-crystallin (20 μM) for 1 h at 37°C. Control samples consisted of buffer alone or αB-crystallin alone. All samples were collected and centrifuged for 30 min at 4°C and the soluble (S) and pellet (P) fractions separated. Pellet fractions were washed twice with PBS and then the soluble and pellet fractions were spotted onto a nitrocellulose membrane in duplicate. Membranes were blotted with antibodies specific to SOD1 or αB-crystallin. The results shown are representative of two independent experiments.

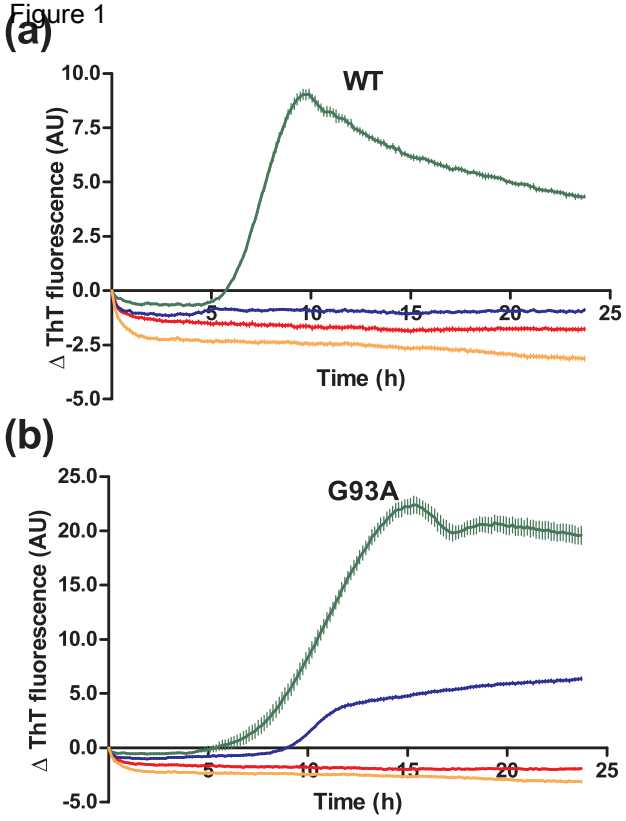

(a)
WT
10.0
7.5
5.0
2.5
0.0
-2.5
-5.0
△ ThT fluorescence (AU)
5
10
15
20
25
Time (h)
(b)
G93A
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
△ ThT fluorescence (AU)
5
10
15
20
25
Time (h)

Figure 1

Figure 2

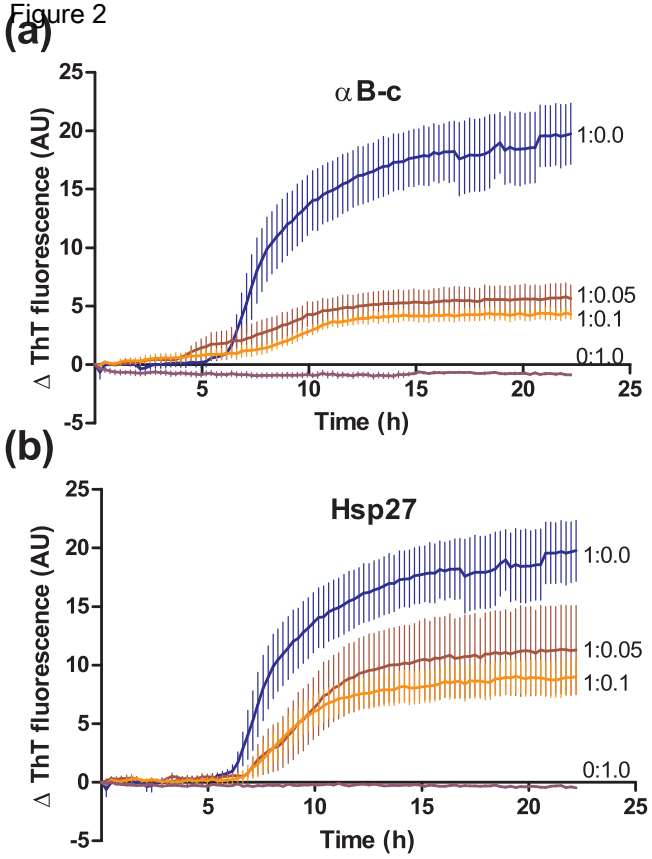


Figure 2

Figure 3

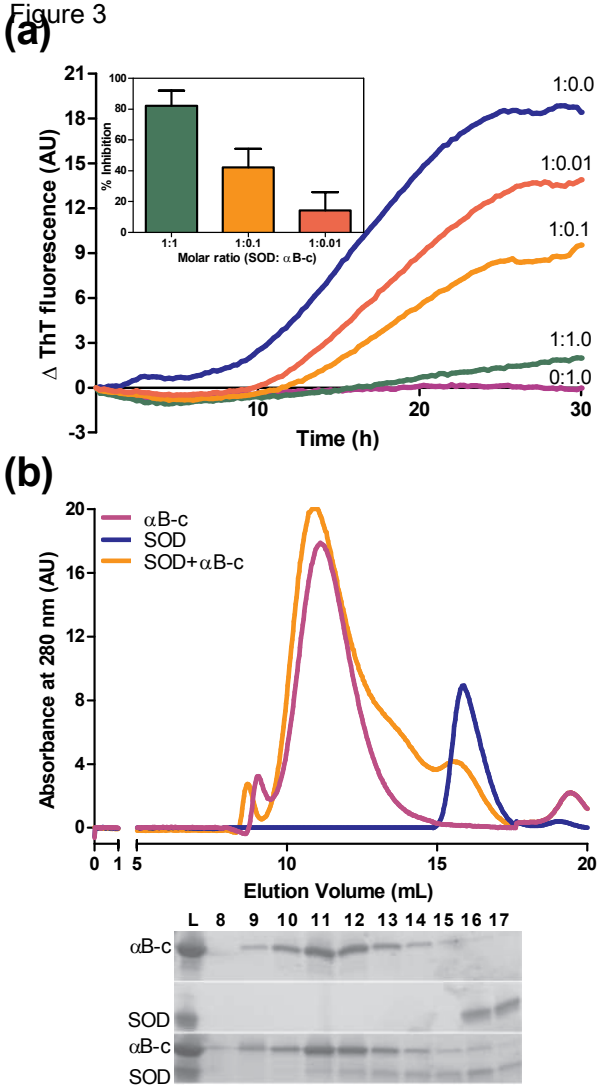
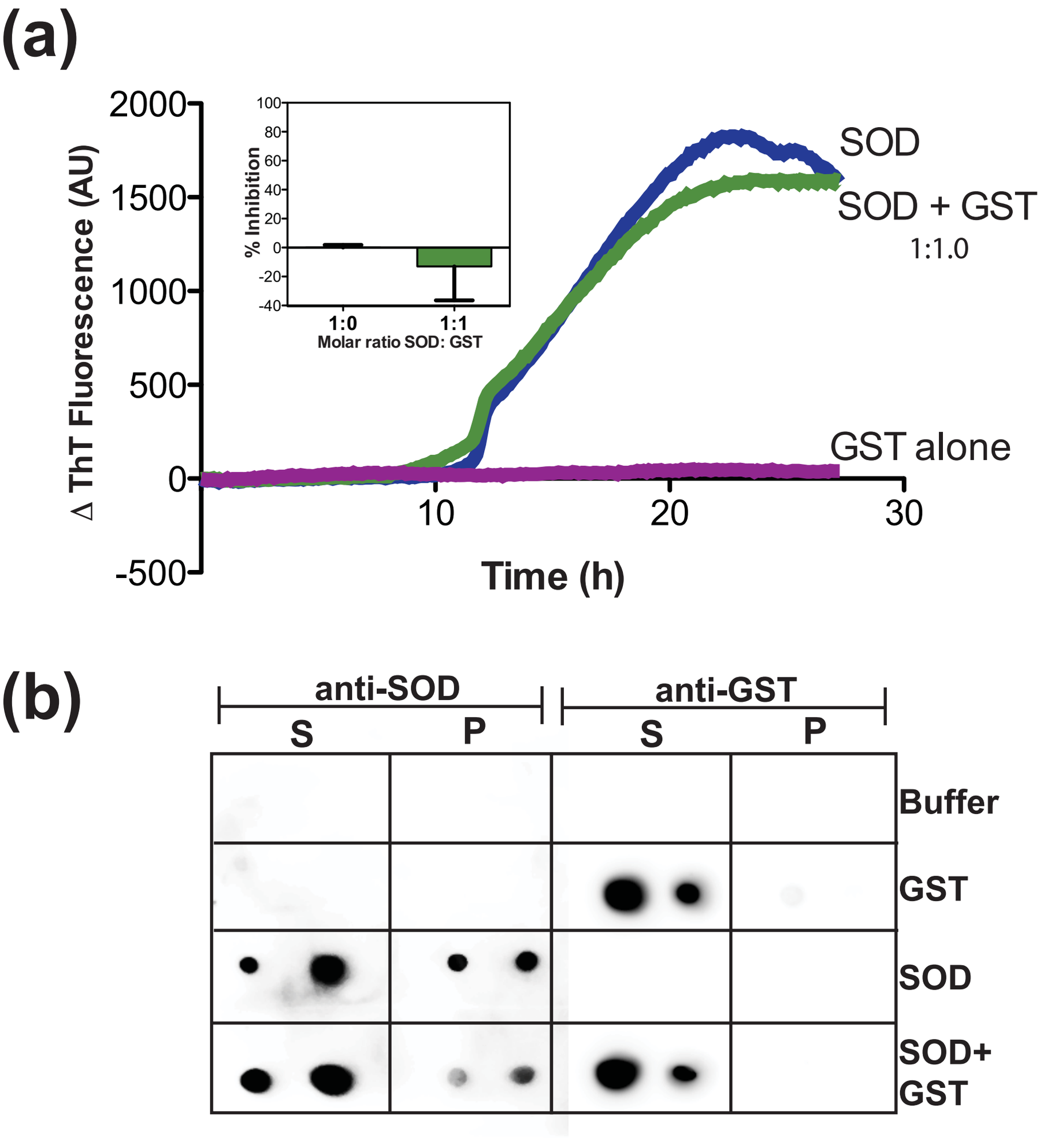


Figure 3

Supplementary Figure 1 –Negative control GST does not inhibit the *in vitro* aggregation of WT SOD1 or form a stable complex with its aggregated form. (a) Wild-type SOD1 was incubated at 30 μM in 10 mM potassium phosphate buffer containing 5 mM EDTA, pH 7.4 whilst shaking (120 rpm for 5 min each cycle, 15 min cycles) at 37ºC in the absence or presence of GST and the amount of ThT fluorescence (excitation at 440 nm and emission at 490nm) was monitored over time using a microplate-reader. The molar ratio (SOD1: GST) used was 1:1. Inset: The percent inhibition of the increase in ThT fluorescence afforded by GST. Results shown are mean ± SEM of three replicates. **(b)** Immuno-dot blot used to detect any interaction of GST with aggregated SOD1. Aggregated WT SOD1 (20 μM) was incubated in the absence or presence of GST (20 μM) for 1 h at 37°C. Control samples consisted of buffer alone or GST alone. All samples were collected and centrifuged for 30 min at 4°C and the soluble (S) and pellet (P) fractions separated. Pellet fractions were washed twice with PBS and then the soluble and pellet fractions were spotted onto a nitrocellulose membrane in duplicate. Membranes were blotted with antibodies specific to SOD1 or GST. The results shown are representative of two independent experiments.


(a)
2000
1500
1000
500
0
-500
Δ ThT Fluorescence (AU)
10
20
30
Time (h)
SOD
SOD + GST
1:1.0
GST alone
100
80
60
40
20
0
-20
-40
% Inhibition
1:0
1:1
Molar ratio SOD: GST
(b)
anti-SOD
anti-GST
S
P
S
P
Buffer
GST
SOD
SOD+
GST