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

Justin J. Yerbury · Dane Gower · Laura Vanags ·
Kate Roberts · Jodi A. Lee · Heath Ecroyd

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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 α B-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. α B-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 Amyotrophic lateral sclerosis · SOD1 · Protein aggregation · Small heat shock proteins · Molecular chaperones · α B-crystallin

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 and 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), increasing its propensity to aggregate into oligomeric forms (Banci et al. 2007), and to form insoluble material in cells (Prudencio et al.

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J. J. Yerbury (✉) · D. Gower · L. Vanags · K. Roberts · J. A. Lee ·
H. Ecroyd (✉)
Illawarra Health and Medical Research Institute,
School of Biological Sciences, University of Wollongong,
Northfields Avenue,
Wollongong, NSW 2522, Australia
e-mail: jyerbury@uow.edu.au
e-mail: heathe@uow.edu.au

2009). Predictions using the Chiti–Dobson equation (that predicts aggregation propensity) have suggested that the protein instability and aggregation propensity are risk factors 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 ageing, 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 neurons 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 α B-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 and Wacker 2005).

The small heat shock proteins (sHsps) α B-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 α B-crystallin increase in abundance in the spinal cord as ALS progresses in mouse models (Vleminckx et al. 2002; Wang et al. 2005, 2008a). α B-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 α B-crystallin antibodies in cell models of SOD1-associated ALS (Shinder et al. 2001). In HEK293 cells, over-expression of α B-crystallin has been shown to suppress mutant SOD1 aggregation (Karch and Borchelt 2010), and α B-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 °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 report that Hsp27 over-expression does not protect Neuro2a cells from SOD1-associated toxicity (Krishnan et al. 2006). In animal models, knockdown of α B-crystallin does not affect the amount of insoluble SOD1 nor does it significantly alter the lifespan of transgenic mice (Karch and Borchelt 2010). Similarly, while over-expression of Hsp27 slows the early phase of disease, it does not alter the lifespan of SOD1 mice (Sharp et al. 2008). This may be, at least in part, due to the fact that robust expression of α B-crystallin and Hsp25 is largely restricted to glial cells. However, there have been no studies that have investigated whether α B-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 α B-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 dithiothreitol (DTT) and ethylenediaminetetraacetic acid (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 wild-type (WT) SOD1 until high concentrations of DTT (50 mM) were used (Fig. 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 (Fig. 1b). This is consistent with the 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 WT SOD1 was very similar to G93A 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.

Fig. 1 The reduction-dependent aggregation of SOD1 in vitro. Wild-type (a) or G93A (b) SOD were incubated at 30 μ M 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 37 $^{\circ}$ C in the absence or presence of 10 mM or 50 mM DTT. The buffer-alone sample is also shown and the concentration of DTT added is given on the right. The amount of ThT fluorescence (excitation at 440 nm and emission at 490 nm) was monitored over time using a microplate reader, and the change in ThT fluorescence is reported as a mean $\pm$ SEM of three replicates. The data are representatives of three independent experiments

Previous studies have reported that, although over-expression of α B-crystallin can reduce insoluble aggregate formation in cell models of SOD1 aggregation, knockdown of α B-crystallin in a G93A SOD1 mouse model of ALS does not affect the total amount of insoluble protein measured (Karch and 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 α B-crystallin and Hsp27. Co-incubation of G93A SOD1 with α B-crystallin resulted in a significant decrease

in ThT fluorescence associated with SOD1 aggregation (Fig. 2a), but it did not alter the lag phase (which remained at $\sim$ 5 h in the presence or absence of α B-crystallin). These results are consistent with α B-crystallin having a direct role in suppressing SOD1 aggregation in cell models over-expressing α B-crystallin. The lack of an obvious change in phenotype upon knockout of α B-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 α B-crystallin in these mice. Alternatively, α B-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 α B-crystallin expression in motor neurons is low in comparison to surrounding glia (Vleminckx et al. 2002), and the initial

Fig. 2 The small heat shock proteins α B-crystallin and Hsp27 inhibit the in vitro aggregation of G93A SOD1. G93A SOD1 was incubated at 30 μ M 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 37 $^{\circ}$ C in the absence or presence of (a) α B-crystallin or (b) Hsp27. The amount of ThT fluorescence (excitation at 440 nm and emission at 490 nm) was monitored over time using a microplate reader, and the change in ThT fluorescence is reported as a mean $\pm$ SEM of three replicates. The data are representatives of at least three independent experiments. Molar ratios (SOD1:chaperone) are shown on the right

stages of SOD1 aggregation occurs in motor neurons in mice (Stieber et al. 2000).

We next tested whether Hsp27 is also able to inhibit G93A SOD1 aggregation *in vitro*. As seen for α B-crystallin, co-incubation of G93A SOD1 with Hsp27 resulted in a decrease in ThT fluorescence associated with SOD1 aggregation (Fig. 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 α B-crystallin (α B-crystallin 78 ± 5 % compared to Hsp27 54 ± 6 %, $p < 0.05$). These data indicate 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 α B-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 α B-crystallin in the insoluble fractions of spinal cord extracts from transgenic mice with ALS symptoms (Wang et al. 2005, 2008a). Therefore, to test if α B-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 α B-crystallin. Aggregation of WT SOD1 when incubated alone was more rapid in this assay than seen previously (compare Fig. 1a with Fig. 3a) due to the increased rate of shaking used for this assay, i.e. 300 revolutions/min (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 α B-crystallin resulted in a concentration-dependent decrease in ThT fluorescence associated with WT SOD1 aggregation, such that, at a molar ratio of 1:1 (SOD1: α B-crystallin); there was nearly complete inhibition (Fig. 3a). The ability to inhibit SOD1 aggregation was specific to α B-crystallin since addition of GST at a 1:1 molar ratio did not significantly affect the aggregation of WT SOD1 in this assay (Supp. Fig1a). Size exclusion chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis (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 (Fig. 3b). α B-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 α B-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 contained both SOD1 and α B-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 α B-crystallin. The formation of a high molecular mass complex between α B-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 and 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 α B-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 Figs. 2 and 3a). If a two-phase kinetic model is used to model the aggregation process of SOD1, these data imply that both Hsp27 and α B-crystallin inhibit SOD1 aggregation by acting primarily upon fibril elongation rather than the formation of fibril nuclei. Thus, our data suggest 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 α B-crystallin has been reported to be capable of binding to preformed fibrils and, in doing so, suppressing further fibril formation (Shammas et al. 2011; Waudby et al. 2010). Moreover, in both humans and mouse models of ALS, α B-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 α B-crystallin is capable of binding to mature SOD1 aggregates using a dot blot assay (Fig. 3c). After centrifugation and washing, a proportion of aggregated SOD1 could be detected in the pellet (P). When aggregated WT SOD1 and α B-crystallin were incubated together, α B-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 α B-crystallin was incubated with this aggregated sample. The ability of α B-crystallin to bind to aggregated forms of SOD1 was specific since GST was not able to bind under the same conditions (Supp. Fig 1b). These results may, at least in part, explain the presence of α B-crystallin in SOD1-positive inclusions in ALS patients and its association with inclusions in mouse models.

Fig. 3 αB-crystallin inhibits the in vitro aggregation of WT SOD1 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 37 °C in the absence or presence of αB-crystallin, and the amount of ThT fluorescence (excitation at 440 nm and emission at 490 nm) was monitored over time using a microplate reader. Molar ratios (SOD1: chaperone) are shown on the right. Inset indicates 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 fractions to establish whether αB-crystallin prevents WT SOD1 aggregation by forming a complex with it. Samples containing non-incubated WT SOD1 (30 μM), αB-crystallin alone (30 μM) or WT SOD1 and αB-crystallin (at a molar ratio of 1:1 and recovered at the end of the assay described in **a**) 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 fivefold with PBS) and every second fraction eluting between 8 and 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 were 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

such as Hsp70) protecting 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 and 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; Stathopoulos 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

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,

which G93A SOD1 evades these systems remains unknown. This study was able to confirm the ability of Hsp27 and α B-crystallin to inhibit G93A SOD1 aggregation in vitro. Since previous studies have suggested that over-expression of chaperones is insufficient to attenuate the progression of 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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