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Authors

Atlasi, Ryan S
Malik, Ravinder
Corrales, Christian I
[et al.](#)

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Investigation of anti-SOD1 antibodies yields new structural insight into SOD1 misfolding and surprising behavior of the antibodies themselves

Ryan S. Atlasi¹, Ravinder Malik¹, Christian I. Corrales¹, Laura Tzeplaeff¹, Julian P. Whitelegge^{2,3,4}, Neil R. Cashman⁵, and Gal Bitan^{1,3,4,*}

¹Department of Neurology, ²Department of psychiatry and biobehavioral sciences, David Geffen School of Medicine, ³Brain Research Institute, and ⁴Molecular Biology Institute, University of California, Los Angeles, CA, USA and ⁵Department of Neurology, University of British Columbia (UBC), Vancouver, BC, Canada

To whom correspondence should be addressed: Gal Bitan, Department of Neurology, David Geffen School of Medicine, UCLA, 635 Charles E. Young Drive South/NRB 451, Los Angeles, CA 90095; gbitan@mednet.ucla.edu; Tel. (310) 206-2082; Fax. (310) 206-1700.

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ABSTRACT

Mutations in Cu/Zn-superoxide dismutase (SOD1) gene are linked to 10–20% of familial amyotrophic lateral sclerosis (fALS) cases. The mutations cause misfolding and self-assembly of SOD1 into toxic oligomers and aggregates, resulting in motor neuron degeneration. The molecular mechanisms underlying SOD1 aggregation and toxicity are unclear. Characterization of misfolded SOD1 is particularly challenging due to its metastable nature. Antibodies against misfolded SOD1 are useful tools for this purpose, provided their specificity and selectivity are well characterized. Here, we characterized three recently introduced anti-misfolded SOD1 antibodies and compared them with two commercial, anti-misfolded SOD1 antibodies raised against the fALS-linked variant G93A-SOD1. As controls, we compared the reactivity of these antibodies to two polyclonal anti-SOD1 antibodies expected to be insensitive to misfolding. We asked to what extent the antibodies could distinguish between WT and variant SOD1 and between native and misfolded conformations. WT, G93A-SOD1, or E100K-SOD1 were incubated under aggregation-promoting conditions and monitored using thioflavin-T fluorescence, electron microscopy, and dot blots. WT and G93A-SOD1 also were analyzed using native-PAGE/Western blot. The new anti-misfolded SOD1 and the commercial antibody B8H10 showed variable reactivity using dot blots, but generally showed maximum reactivity at the time misfolded SOD1 oligomers were expected to be most abundant. In contrast, only B8H10 and the control antibodies were reactive in Western blots. Unexpectedly, the polyclonal antibodies showed strong preference for the misfolded form of G93A-SOD1 in dot blots. Surprisingly, anti-misfolded SOD1 antibody C4F6 was specific for the apo form of G93A-SOD1 but insensitive to misfolding. Antibody 10C12 showed preference for early misfolded structures, whereas 3H1 bound preferentially to late structures. These new antibodies allow distinction between putative early- and late-forming pre-fibrillar SOD1 oligomers.

ALS is a progressive neurodegenerative disease that causes death of motor neurons. In most cases, the life expectancy for patients with ALS is 3–5 years after diagnosis.¹ Approximately 10% of ALS cases are familial, and of those, 10–20% are associated with >180 mutations in the Cu/Zn Superoxide Dismutase (SOD1) cognate gene (<http://alsod.iop.kcl.ac.uk>). SOD1 is a homodimeric, antioxidant enzyme that catalyzes highly reactive superoxide radical anions formed during respiration into less reactive hydrogen peroxide. Each monomer complexes one Cu²⁺ and one Zn²⁺ ion, which are essential for the structural stability and catalytic activity of SOD1,² and contains a highly conserved intramolecular disulfide bond. Reduction of the disulfide bond and/or loss of the metal ions destabilize the structure of SOD1 leading to its misfolding and self-assembly into toxic oligomers and aggregates.^{3,4}

Multiple studies have characterized the aggregation mechanism, kinetics, and fibril morphology of SOD1,⁵⁻⁷ whereas characterization of SOD1 oligomers has been more challenging because of their

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3 metastable nature. The oligomers are short-lived, exist in dynamically changing mixtures, and their
4 structures are influenced by the method of preparation. Antibodies specific for the oligomeric and
5 misfolded structures of amyloidogenic proteins are useful tools for identifying these structures in
6 biological samples,⁸⁻¹⁰ though they should be used with caution due to potential low specificity and cross-
7 reactivity.^{11, 12} Antibodies against misfolded SOD1 have been prepared taking advantage of knowledge of
8 the three-dimensional structure of the dimeric protein and using epitopes buried in the dimer interface.¹³
9 However, information about the specificity of many antibodies is limited and, in many cases, the exact
10 epitope and binding characteristics are not validated. Unreliability and insufficiently characterized
11 antibodies have been a major impediment for data reproducibility in biomedical research, including ALS
12 research.¹⁴⁻¹⁶
13

14 To address these issues, here, we characterized three new anti-misfolded-SOD1 antibodies¹⁷ and
15 compared them with two commercial, anti-misfolded-SOD1 antibodies raised against the fALS-linked
16 variant G93A-SOD1. We compared the reactivity of these antibodies to two polyclonal anti-SOD1
17 antibodies that were expected to be insensitive to misfolding (Table 1). We investigated whether the
18 antibodies could distinguish among native and misfolded forms of SOD1 and how their binding was
19 affected by SOD1 self-assembly. Interestingly, the results revealed that the behavior of several antibodies
20 did not match their expected reactivity. In addition, the different temporal change in antibody reactivity
21 relative to the increase in thioflavin T (ThT) fluorescence showed formation of early and late SOD1
22 structures, presumably oligomers, which to our knowledge, have not been reported previously.
23
24

25 RESULTS AND DISCUSSION

26 *SOD1 aggregation is highly variable and includes two distinct structural change steps.* To create a
27 baseline to which characterization of anti-SOD1 antibodies could be related, the aggregation process first
28 was monitored for 40 h using the ThT fluorescence assay, which measures formation of the typical cross-
29 β structure of amyloids.¹⁸ To facilitate the reaction, we used the de-metallated, apo form of WT or G93A-
30 SOD1 and induced the aggregation by addition of 25 mM DTT.⁵ In control reactions, apo-SOD1 was
31 incubated under identical conditions in the absence of DTT. Because the kinetics of unseeded SOD1
32 aggregation has been reported to be highly variable,¹⁹⁻²¹ multiple reactions were monitored in parallel in
33 96-well plates using a plate reader.
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36 As expected, under non-reducing conditions, the ThT fluorescence signal for WT or G93A-SOD1 showed
37 little change during the 40-h incubation (Figure 1A, B), suggesting minimal misfolding and aggregation.
38 In contrast, in the presence of DTT (Figure 1C, D), in all of the reactions we observed a substantial
39 increase in the ThT fluorescence signal, consistent with misfolding and formation of β -sheet-rich
40 amyloid. This behavior was more prominent in G93A-SOD1 (Figure 1D) than in WT SOD1 (Figure 1C),
41 in agreement with the increased tendency of G93A-SOD1 to aggregate. Interestingly, inspection of the
42 ThT curves revealed a two-phase process in most cases (Figure 1C, D insets). After an initial lag phase
43 (L), the ThT fluorescence showed a small increase (I1) followed by an intermediary plateau or “pause”
44 (P1). Then, a second, large increase in ThT fluorescence (I2) was observed, culminating in a final plateau
45 (P2). A similar behavior has been observed previously in aggregation reactions of N139D-SOD1 and the
46 triply substituted variant N26D/N131D/N139D-SOD1.²² Another study measuring the aggregation
47 kinetics of WT SOD1 in the presence of small peptides with high aggregation propensity derived from the
48 sequence of WT SOD1 showed similar, two-step ThT curves, but only in the presence of the peptide
49 inducers.⁷ To our knowledge, our study is the first one to observe this behavior for WT SOD1 itself or for
50 G93A-SOD1. A possible explanation for this unusual behavior was the high speed of agitation of the
51 samples, which was imposed by the available settings of the plate reader used in our experiments, a
52 Biotek Synergy HT, in which the slowest agitation setting is 1,020 rpm. To test if this was the case, we
53 performed similar reactions in a newer model, Biotek Synergy HTX, at an agitation speed of 300 rpm.
54 Under these conditions, we did not observe an increase in ThT fluorescence during the same incubation
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3 time. However, when we added Teflon balls to the wells, which were not included in the reactions
4 performed in the older instrument, the ThT fluorescence did increase and in some reactions, we observed
5 a two-step profile (Supplementary Figure 1), similar to the one shown in Figure 1C,D, suggesting that this
6 behavior was an intrinsic feature of SOD1 aggregation and not restricted to a particular experimental
7 setting. Presumably, under the conditions we used, the misfolding of native SOD1 and subsequent
8 nucleation step were accelerated, but elongation initially was suppressed leading a “pause” in the reaction
9 and observation of the intermediate plateau phase, P1. However, the exact mechanism controlling the
10 two-step kinetic profile is not clear and will require further study.
11

12 Examining the behavior of different replicates, it was evident that the length of the different steps varied
13 substantially. An exception was the initial lag phase, which was fairly consistent among different
14 replicates and was shorter for WT SOD1 (~1.5 h) than for G93A-SOD1 (~4 h) (*cf.* Figure 1C, D). This
15 trend was reversed in the experiments shown in Supplementary Figure S1, which were done later, further
16 demonstrating the stochastic nature of the process. A literature search showed that lag times of fALS-
17 related SOD1 variants that were longer than that of WT SOD1 have been reported previously^{23, 24}. The
18 differences may be related to small variations in experimental conditions and possibly residual metal-ion
19 content of the proteins. The fluorescence values and duration of the other steps of the ThT curves were
20 highly variable. For example, the final plateau values were in the range 200–1,250 for WT SOD1 and
21 370–2,600 for G93A-SOD1. Nearly 30% of the WT SOD1 aggregation reactions reached the final
22 plateau, whereas > 90% of the G93A-SOD1 aggregation reactions reached the final plateau,
23 demonstrating the accelerated aggregation of the latter (Figure 1C, D).
24

25 *SOD1 forms a mixture of amorphous and fibrillar assemblies.* To examine the correlation between the
26 aggregation kinetics and morphology of SOD1, separate aggregation reactions were conducted as
27 described in the Materials and Methods. Aliquots of these reaction mixtures were taken at different time
28 points during the aggregation reaction and were used to measure ThT fluorescence (not shown) and in
29 parallel for morphological examination by EM (Figure 2). Initially, (t = 0 h), no protein was observed,
30 suggesting that only SOD1 monomers or dimers, which are too small to be observed under the conditions
31 used, were present (Figure 2A, F). At 12 h, corresponding in most cases to the P1 phase observed in ThT
32 experiments, small ring-like aggregates were observed in the WT protein (Figure 2B) whereas G93A-
33 SOD1 formed amorphous structures (Figure 2G). At t = 16 h, a later time point during the P1 phase in
34 most reactions, the morphology of WT SOD1 changed to small amorphous aggregates connected by
35 thread-like structures (Figure 2C). Similar morphologies also were observed for G93A-SOD1, in addition
36 to short fibrils (Figure 2H). At 28 h, during a period in which most reactions reached the final plateau, P2,
37 a large mesh of amorphous threads, which were thinner in WT SOD1 and thicker in G93A-SOD1, was
38 observed (Figure 2D, I), in addition to occasional fibrils. Finally, at ~168 h (1 week), large amorphous
39 aggregates and occasional fibrils integrated within them were observed (Figure 2E, J). Overall, the
40 majority of the morphologies observed were amorphous, rather than fibrillar, despite the high ThT signal,
41 suggesting that under the conditions used here, SOD1 largely was arranged in β -sheets but the structures
42 were not classical amyloid.
43

44 *Characterization of conformational changes during SOD1 aggregation using dot blots with anti-SOD1*
45 *antibodies.* To set a baseline for antibody reactivity, we analyzed first the behavior of the antibodies with
46 WT- and G93A-SOD1 incubated in the absence of DTT. Under these conditions, we expected SOD1 to
47 maintain primarily a native-like dimeric structure, an expectation supported by the minimal change in
48 ThT fluorescence observed during the experiment (Figure 1A, B and Figure 3E, F). Thus, we predicted
49 that under these conditions only the antibodies that were not specific for misfolded SOD1, Ab16831 and
50 2770^{17, 25, 26} would bind to SOD1.
51

52 To characterize the antibody binding to SOD1, aliquots of the reaction mixtures were taken every 4 h
53 during the same aggregation reactions used for ThT fluorescence monitoring, and deposited onto
54 nitrocellulose membranes for dot-blot analysis. The membranes then were probed with each of the seven
55 antibodies, the dots were quantified densitometrically, and the reactivity of each antibody was plotted
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3 against time. To avoid a systematic error due to potential gradual loss of material with multiple strip/re-
4 probe cycles, the order of the antibodies was changed for different membranes. In separate experiments,
5 the metallated version of each SOD1 variant was probed as a control.
6

7 The two pAbs, Ab16831 and 2770, indeed showed high reactivity with both WT (Figure 3A, C, here, and
8 in all subsequent figures, the lines are meant to help guide the eye, not to suggest that the dots should be
9 connected) and G93A-SOD1 (Figure 3B, D). They showed approximately the same reactivity with WT
10 SOD1, whereas the reactivity of 2770 with G93A-SOD1 was significantly higher than that of Ab16831 (p
11 < 0.0001 , 2-way ANOVA). Interestingly, this high reactivity of 2770 with G93A-SOD1 was only with the
12 apo form, whereas low reactivity was observed with the metallated form (Figure 3B). There was no
13 difference between the reactivity of 2770 with the metallated and apo forms of WT SOD1, or between the
14 reactivity of Ab16831 with the apo and metallated forms of either SOD1 isoform.
15

16 Surprisingly, the anti-misfolded SOD1 mAbs B8H10²⁷⁻²⁹ and AMF7-63²⁸ also bound to both isoforms of
17 SOD1 under non-reducing conditions, regardless of metallation (Figure 3A–D). Previously, it has been
18 suggested that filter binding might partially denature SOD1, potentially inducing exposure of the
19 disordered electrostatic loop recognized by the high-affinity antibody AMF7-63, but not by the lower-
20 affinity mAb 3H1, which was raised against the same antigen. In support of this interpretation, AMF7-63
21 was reported not to bind to spinal cord sections of transgenic mice expressing human, WT SOD1²⁸.
22

23 As expected, mAb 3H1^{17, 30} showed very weak binding to the apo form of WT or G93A-SOD1 and no
24 binding to the metallated form. mAb 10C12^{17, 30} did not bind at all to either SOD1 isoform regardless of
25 metallation. Interestingly, C4F6, which was expected to bind only misfolded SOD1^{29, 31, 32} did not bind at
26 all to WT SOD1 (Figure 3A, C) or to metallated G93A-SOD1, but had the highest apparent reactivity for
27 apoG93A-SOD1 (Figure 3B, D), suggesting that it is specific for apoG93A-SOD1 and not for misfolded
28 SOD1. As expected, under the non-reducing conditions used in these experiments, most of the antibodies
29 bound to both WT and G93A-SOD1 with little change in apparent reactivity during the incubation. To
30 determine whether a change in reactivity occurred, we used a 1-way ANOVA to calculate whether the
31 reactivity at each time point differed significantly from the initial or final time points. The antibodies for
32 which significant change was found are shown in filled symbols, whereas those that showed no
33 significant change are shown in empty symbols (Figures 3C, D; 4C, D; and 5C, D). The analysis showed
34 that the reactivity of Ab16831 increased significantly and then decreased during the incubation of both
35 SOD1 isoforms, and that the reactivity of both AMF7-63 and 3H1 decreased during the incubation of WT
36 SOD1. However, these changes were relatively small in magnitude and may reflect experimental
37 variability.
38

39 We also compared the reactivity of the antibodies to each other using a 2-way ANOVA. The analysis
40 showed that the antibodies could be divided into groups, with significant differences among the groups,
41 and little or insignificant differences within each group. In the case of WT SOD1, a low-reactivity group
42 consisted of C4F6, 3H1, and 10C12. AMF7-63 showed medium reactivity, whereas a high-reactivity
43 group comprised Ab16831, 2770, and B8H10. In the case of G93A-SOD1, mAbs 3H1 and 10C12 were in
44 the low-reactivity group, whereas C4F6 moved to the high-reactivity group, together with pAb 2770.
45 Ab16831, B8H10, and AMF7-63 comprised the medium-reactivity group.
46

47 Next, we asked whether the reactivity of the antibodies changed during misfolding and self-assembly of
48 SOD1 under reducing conditions. Because of the high variability in the ThT behavior under these
49 conditions (Figure 1), and because the “pause” period, P1, was the time when the highest activity was
50 observed for most of the antibodies, we divided the different aggregation reactions into two groups
51 defined by the duration of P1 (t_{P1}). Group 1 included reactions with a short P1, $t_{P1} \leq 10$ h, comprising
52 ~75% of the WT SOD1 reactions, and $t_{P1} \leq 7$ h, comprising ~50% of the reactions of G93A-SOD1
53 (Figure 4). Group 2 comprised the remaining reactions with $t_{P1} > 10$ h for WT SOD1 (~25% of the
54 reactions) and $t_{P1} > 7$ h for G93A-SOD1 (~50% of the reactions, Figure 5). In each Figure, panels E and F
55 show an example of one ThT profile, which was chosen with particularly short P1 periods in Figure 4 and
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3 long P1 periods in Figure 5 to demonstrate the high variability in kinetics. The dot-blot graphs in panels C
4 and D are averages of multiple reactions and should not be compared directly to the single ThT examples.

5
6 Under these conditions, all the antibodies, except C4F6, showed an initial increase in reactivity with WT
7 SOD1 followed by a decline. This change was statistically significant for the reactivity of all antibodies
8 with WT SOD1 except for pAb 2770 (Figures 4C, 5C). AMF7-63 showed statistically significant change
9 in slow reactions (Figure 5C), but not fast reactions (Figure 4C). C4F6 did not react with WT SOD1,
10 similar to the results under non-reducing conditions. Under these conditions, only the unreactive C4F6
11 and highly reactive 10C12 differed significantly from the other antibodies, whereas the reactivities of
12 Ab16831, 2770, B8H10, AMF7-63, and 3H1 did not differ significantly from each other (Figures 4C,
13 5C).

14
15 In the case of G93A-SOD1, all the antibodies, including C4F6 showed a significant change in reactivity
16 during the aggregation reaction (Figure 4D). The change was smaller for mAbs 3H1 and C4F6 than for
17 other antibodies. 3H1 reached maximal reactivity at 12 h, later than most other antibodies, which showed
18 maximal reactivity between 4–8 h (Figure 4D). C4F6 had its maximal reactivity between 0–4 h and then
19 the signal declined to about 50% of the original reactivity by 24 h and did not change at later time points.
20 The ~2-fold change in C4F6 was the smallest of all the antibodies tested, supporting the notion that it was
21 specific for the apo form of G93A-SOD1 variant itself and was affected to a small degree by
22 conformational change.

23
24 Comparison of antibody reactivities showed again three groups in the fast reactions (Figure 4D), which
25 differed significantly from each other. A low-reactivity group comprising C4F6 and 3H1, a medium-
26 reactivity group consisting of Ab16831, B8H10, and AMF7-63, and a high-reactivity group including
27 2770 and 10C12. In the medium-reactivity group, AMF7-63 deviated significantly from Ab16831 and
28 B8H10 at later time points, in which its reactivity remained relatively high compared to the other two
29 antibodies. In slow reactions (Figure 5D), the reactivity of most antibodies was somewhat higher than in
30 the fast reactions, leading to a shift of antibodies Ab16831, B8H10, and AMF7-63 to the high reactivity
31 group, and separation between C4F6, which remained the one with the lowest reactivity, and 3H1, which
32 displayed medium reactivity (Figure 5D).

33
34 Interestingly, the reactivity of C4F6 with G93A-SOD1 under reducing conditions (Figures 4, 5) was
35 $\leq 50\%$ of its reactivity under non-reducing conditions (Figure 3). Importantly, both conditions were
36 blotted and developed on the same membranes allowing side-by-side comparison. These results suggested
37 that the antibody recognizes an epitope containing A93 in the folded conformation of the protein and
38 actually loses some of its reactivity when G93A-SOD1 misfolds and aggregates.

39
40 The reactivity of most of the other antibodies was substantially higher for G93A-SOD1 than for WT
41 SOD1. The one exception was mAb 10C12, which showed high reactivity with both forms of SOD1. In
42 the case of WT SOD1, the maximal reactivity of 10C12, at $t = 8\text{--}12$ h, was 2–4 times higher than that of
43 all other antibodies. As shown in Figure 5, this peak in reactivity corresponded approximately to the
44 beginning of the P1 phase in the ThT curve. In reactions of WT SOD1 with a short P1 phase (Figure 4A,
45 C), most of the antibodies also showed maximal reactivity around the same time, but their reactivity was
46 substantially lower than that of 10C12. mAbs AMF7-63, 3H1, and 10C12 showed consistently a
47 significant change in reactivity over the course of the aggregation reaction with WT SOD1, regardless of
48 the kinetics of individual reactions. In contrast, the change was statistically significant for Ab16831 and
49 B8H10 in reactions with a short P1 (Figure 4C), but not in those with a long P1 (Figure 5C). The
50 reactivity of pAb 2770 did not change significantly during the aggregation of WT SOD1.

51
52 mAb 10C12 was raised against a form of the SOD1-exposed-dimer-interface (SEDI) antigen containing a
53 cysteic acid residue,¹³ which raised the concern that it might have bound to an inadvertently oxidized
54 form of SOD1. To test if this were the case, we examined the proteins by MS. These experiments were
55 done in the presence of DTT to facilitate reduction of disulfide bonds. However, if cysteic acid existed in
56 the protein, it could not have been reduced by DTT.³³ The observed masses of WT and G93A-SOD1,
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3 15,848 and 15,863, respectively, corresponded to the intact polypeptides bound to two Na⁺ ions
4 (calculated masses, 15,847 and 15,861, respectively). We did not observe oxidized forms of either WT or
5 G93A-SOD1 in these experiments (Figure 6). To further test if a minor population of the protein
6 contained cysteic acid, we incubated WT- or G93A-SOD1 with dimedone, a reagent used to label sulfenic
7 acids. We did not detect a change in the molecular weight of either protein following incubation with
8 dimedone (data not shown), suggesting that cysteic acid was not present in the proteins we used and did
9 not contribute to mAb 10C12 binding. The reactivity of the antibody, thus, likely was against the
10 unoxidized form of the SEDI region.
11

12 In the aggregation reactions of G93A-SOD1, all the antibodies showed significant changes in reactivity,
13 regardless of kinetics (Figures 4D and 5D). In reactions with a short P1 phase, pAb 2770 behaved
14 similarly to 10C12 (Figure 4B, D). Both antibodies had the highest reactivity for G93A-SOD1 and both
15 displayed 5-times higher reactivity at ~8 h, approximately correlating with the P1 phase in the ThT curve,
16 than at the end of the reaction. Reactions with longer P1 showed that the peak in reactivity of 10C12
17 actually was the earliest, at ~12 h, whereas the peak in reactivity of other antibodies was at 16–20 h and
18 the latest one to peak was mAb 3H1 (Figure 5B, D). Interestingly, although the reactivity of all the
19 antibodies started to decrease at ~12 h in reactions of G93A-SOD1 with a short P1 (Figure 4B, D) or
20 ~16–20 h in reactions with a long P1 (Figure 5B, D), concomitantly with the second increase in ThT
21 fluorescence (Figures 4F, 5F), the reactivity continued to decrease after the ThT fluorescence reached the
22 final plateau, P2 (~16 h in Figure 4F and ~28 h in Figure 5F), suggesting that additional conformational
23 changes and/or self-association took place during that period. These changes were not reflected in the
24 ThT fluorescence, but possibly were associated with the morphological changes observed in Figure 2.
25 Antibodies AMF7-63 and 3H1 were less sensitive to this change than the other antibodies, suggesting that
26 the common antigen of these antibodies (Table 1) becomes only partially shielded at the time β -sheet-rich
27 aggregates formed.
28

29 The observed reactivity of the antibodies with the different forms of SOD1 is summarized in Table 2.
30 Taken together, the data suggested that of the three new antibodies, mAb 10C12 was the most sensitive
31 for misfolded WT SOD1. Its sensitivity and selectivity exceeded not only those of the other new mAbs,
32 3H1 and AMF7-63, but also of the commercial mAb B8H10. In the case of G93A-SOD1, 10C12 showed
33 similar sensitivity and selectivity for misfolded SOD1, with a preference for early forming oligomers.
34 mAbs 3H1 and AMF7-63 also were sensitive and selective for misfolded G93A-SOD1. The peak in
35 reactivity followed the order 10C12 (~12 h), AMF7-63 (~16 h), and finally 3H1 (~20 h, Figure 5D).
36 However, as discussed above, the latter two antibodies had lower selectivity between oligomers and later
37 forming β -sheet-rich aggregates than 10C12 or the commercial antibodies. These results suggest that at
38 least three states can be distinguished by the panel of antibodies we used – early, intermediate, and late
39 oligomers. To our knowledge, these different oligomeric states are reported here for the first time. It will
40 be highly interesting to attempt to isolate them and study the structural differences among them in the
41 future.
42

43 The commercial antibody B8H10 behaved similarly to AMF7-63 during the aggregation reactions,
44 showing moderate reactivity with little change over time for WT SOD1, but a strong sensitivity and
45 selectivity for misfolded G93A-SOD1, with a peak at ~16 h and little reactivity for β -sheet-rich
46 aggregates. As discussed above, antibody C4F6 bound only to G93A-SOD1 but showed little change in
47 reactivity with misfolding or aggregation. A highly surprising result was the behavior of the polyclonal
48 antibodies, Ab16831 and 2770 with G93A-SOD1. Both antibodies showed clear selectivity for the
49 misfolded form of G93A-SOD1 protein, and little change in reactivity during the aggregation of WT
50 SOD1, similar to B8H10, AMF7-63, and 3H1.
51

52 *Characterization of the reactivity of anti-SOD1 antibodies using native-PAGE/Western blot.* To gain
53 further insight into the changes in SOD1 assembly state and the reactivity of the antibodies over time, we
54 used native PAGE and Western blots. Aliquots of the reaction mixtures were fractionated by native
55 PAGE, transferred to nitrocellulose membranes, and probed sequentially with the seven antibodies. As
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3 was done in the dot-blot experiments, the order of the antibodies was changed for each of three replicate
4 membranes to avoid potential loss of signal during strip/re-probe cycles.

5
6 Interestingly, the new mAbs, AMF7-63, 3H1, and 10C12 showed no reactivity at all in the Western blot
7 experiments. The two pAbs, Ab16831 and 2770, reacted with both WT SOD1 and G93A-SOD1 and
8 revealed changes in assembly state over time (Figure 7). mAb B8H10 showed a similar reactivity pattern
9 to the two pAbs, whereas C4F6 had very weak reactivity with G93A-SOD1 only (data not shown).
10 Ab16831, 2770, and B8H10 had a similar staining pattern and all three antibodies showed marked
11 differences between WT SOD1 and G93A-SOD1.

12
13 Interestingly, these three antibodies had low reactivity with non-reduced (NR) WT SOD1 or with reduced
14 SOD1 at $t = 0$ h, whereas at the next time point, $t = 4$ h, the reactivity increased substantially. The blots
15 showed two main bands, likely corresponding to native SOD1 dimer and to the monomer generated by its
16 dissociation. The mobilities of these bands corresponded to higher molecular weights than those of SOD1
17 monomer and dimer (calculated MW = 15.8 and 31.6 kDa, respectively), which is typical for native
18 PAGE. Additional bands between the monomer and dimer and below the monomer presumably
19 corresponded to minor alternative conformations or degradation products. The intensity of the dimer band
20 appeared to reach a maximum at 16 and 20 h when probing with Ab16831 (Figure 7A) and B8H10 (Figure
21 7E), but not when probing with 2770 (Figure 7C). At later time points, the intensity of both bands
22 decreased gradually. The dimer band decreased more rapidly, consistent with its dissociation into
23 monomers. Starting at $t = 20$, high-molecular-weight protein, which did not enter the gel, was observed at
24 the bottom of the wells, suggesting formation of aggregates, in agreement with the ThT (Figure 1C) and
25 EM (Figure 2) data. Concomitant with these changes, the intensity of the two bands with the fastest
26 mobility increased gradually. We believe that these bands may represent alternative conformations of monomeric
27 SOD1. We cannot rule out that they were degradation products, but their recognition by B8H10 suggests
28 against this interpretation.

29
30 The pattern observed with G93A-SOD1 was distinct. A strong dimer band was observed in the NR lane
31 with Ab16831 (Figure 7B) and 2770 (Figure 7D), and a monomer band appeared immediately following
32 addition of DTT at $t = 0$ h. These bands also were detected by B8H10, but their reactivity with this
33 antibody was much weaker, in agreement with its characterization as selective for misfolded SOD1. At 4
34 h, a high-intensity smeary band appeared, likely corresponding to multiple conformers of the monomer,
35 and a higher molecular weight smear was observed above it, presumably representing G93A-SOD1
36 dimer, trimer, and possibly higher molecular weight oligomers. With time, the lower band's intensity
37 decreased and the higher band's intensity increased, consistent with their assignment as monomer and
38 oligomers, respectively. Probing with Ab16831 (Figure 7B) or B8H10 (Figure 7F), but not 2770 (Figure
39 7D), revealed that between 12–20 h, the apparent molecular weight of the smeary oligomer band
40 increased somewhat. At the same time, high-molecular-weight putative protein aggregates at the bottom
41 of the well were observed in blots probed by Ab16831 or 2770, but not B8H10.

42
43 Overall, the native-PAGE/Western-blot data demonstrated different behaviors of WT- and G93A-SOD1.
44 As expected, the disease-associated variant converted into putative insoluble, high-molecular weight
45 aggregates substantially faster than the WT protein and the G93A-SOD1 intermediates forming along the
46 way were smearier and less distinct than those of WT SOD1. Presumably, although native PAGE is a
47 mild, non-denaturing electrophoretic method, unlike dot blots it is sufficient to induce conformational
48 changes precluding recognition of any form of SOD1 by AMF7-63, 3H1, and 10C12, and to a large
49 extent also of G93A-SOD1 by C4F6.

50
51 *Examination of E100K-SOD1 by dot blots.* In view of the surprising behavior of the antibodies, we asked
52 if the pattern of the reactivity against mutant SOD1 was specific for the G93A variant. To address this
53 question, we tested the reactivity of the antibodies using the dot-blot paradigm against a variant
54 containing an amino-acid substitution close to G93A – E100K-SOD1. In this case, dot-blots were
55 obtained every 8 h, rather than 4h, but other aspects of the experiment were similar. Six replicate
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3 reactions were analyzed, showing a similar behavior to G93A-SOD1 (Figure 8A). The lower absolute
4 ThT fluorescence levels in experiments with E100K-SOD1 (*cf.* Figure 1D and 7A) were due to different
5 instrument settings. Four of the reactions (green, red, purple, and orange curves in Figure 8A) followed
6 highly similar courses and therefore the quantitative dot-blot analysis included only these four reactions.
7 The behavior of most antibodies toward E100K-SOD1 was similar to their reactivity with WT SOD1
8 (Figure 8B,C). Thus, Ab16831, 2770, and B8H10 showed relatively high reactivity already at $t = 0$ and
9 their reactivity decreased by up to 2-fold with aggregation. AMF7-63 showed a similar pattern but had
10 lower reactivity toward E100K-SOD1 than the former three antibodies. Antibody 10C12 showed no
11 reactivity at $t = 0$, but a strong reactivity peak at $t = 8$, followed by a sharp decline in reactivity at later
12 time points. Surprisingly, 3H1 behaved similarly to C4F6 in this case and showed no reactivity with
13 E100K-SOD1 regardless of aggregation. These observations supported the notion that C4F6 was specific
14 for G93A-SOD1 and not for a misfolded form of the protein, and suggested that despite being raised
15 against the same peptide epitope, SOD1(125–142), antibodies 3H1 and AMF7-63 differ not only in
16 reactivity, but also in the actual three-dimensional epitope they bind to.

17
18 Another surprising finding was that the commercial mAbs, B8H10 and C4F6 had different selectivity
19 profiles than those previously reported.^{27-29, 31, 32} Although B8H10 showed a peak in reactivity followed by
20 a decline during the aggregation of SOD1, which was more prominent in the case of the G93A and
21 E100K variants (Figure 4, 5, 7) than the WT protein, this mAb also showed high reactivity with
22 unaggregated SOD1 incubated under non-reducing conditions (Figure 3). This kind of behavior was
23 expected, and indeed was confirmed, for Ab16831 and 2770, but not for B8H10. Nonetheless, the mAb
24 reacted with WT SOD1 under non-reducing conditions at the same level as the two pAbs (Figure 3A, C).
25 In the case of G93A-SOD1, the reactivity of B8H10 under non-reducing conditions was similar to that of
26 Ab16831, whereas 2770 showed higher reactivity (Figure 3B, D). These three antibodies actually showed
27 lower reactivity for WT SOD1 under reducing conditions than under non-reducing conditions (*cf.* Figure
28 3 with Figures 4, 5). Taken together, the data suggest that mAb B8H10 reacts with both folded and
29 misfolded SOD1 and is not more specific for misfolded SOD1 than antibodies Ab16831 or 2770.

30
31 The most surprising behavior was of antibody C4F6, which showed no reactivity with WT or E100K-
32 SOD1 regardless of folding/misfolding, strong reactivity with apoG93A-SOD1 under non-reducing
33 conditions (the strongest of the seven antibodies tested), weaker reactivity under reducing conditions, and
34 little change with oligomerization and aggregation (Figures 3–5,7). C4F6 did not react with metallated
35 G93A-SOD1 (Figure 3B). Thus, our data suggest that this antibody is specific for an epitope in G93A-
36 SOD1 that does not exist in WT or E100K-SOD1, is obscured in the metal-bound form of this variant,
37 and is partially shielded under reducing conditions, regardless of misfolding or self-assembly of G93A-
38 SOD1.

39
40 In summary, by monitoring the aggregation of WT and disease-associated SOD1 forms using ThT
41 fluorescence, EM, dot blots, and native-PAGE/Western blots with seven different antibodies, we provide
42 evidence for the existence of at least three intermediate structures that form and disappear during the self-
43 assembly process of the protein under reducing conditions. Distinction of these intermediates has not been
44 reported previously and may lead to identification of potential targets for therapeutic intervention. In
45 addition, our data indicate that currently used commercial antibodies have different specificity and
46 selectivity profiles than previously reported and interpretation of data obtained using these antibodies
47 should be exercised with caution.

48 49 METHODS

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51 *Protein Expression and Purification.* Human, WT, G93A-, and E100K-SOD1 were expressed in
52 *Saccharomyces Cerevisiae* and purified by sequential chromatography according to published protocols.³⁴
53 Part of each protein preparation was de-metallated using EDTA by dialysis as described previously.^{5, 34}
54 Metallated and de-metallated proteins were flash-frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$.
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3 *ThT Fluorescence Assay.* Reaction mixtures containing 10 mM potassium phosphate, 40 μ M WT, G93A-,
4 or E100K-SOD1 were prepared in the absence (WT and G93A-SOD1 only) or presence (all three
5 isoforms) of 25 mM DTT. All solutions were made freshly and filtered through 0.2- μ m filters. The
6 reactions were conducted in two formats: For comparison between ThT fluorescence and dot blots
7 (Figures 1, 3–5, and 7) the reactions were carried out in white Nunc™ 96-Well Optical-Bottom plates
8 with a polymer base (Thermo Fisher), which were agitated using an elliptical motion at 1,020 rpm. Each
9 well contained 40 μ M ThT and fluorescence was monitored every 15 minutes in a BioTek Synergy HT
10 Multi-Detection Microplate Reader at $\lambda_{\text{ex}} = 420$ nm (slit width = 50 nm) and $\lambda_{\text{em}} = 485$ nm (slit width = 20
11 nm). For comparison with electron microscopy (EM, Figure 2, WT and G93A-SOD1 only), reactions
12 were performed in the absence of ThT because control reactions containing no protein showed ThT
13 crystals on the EM grids. One-mL reaction mixtures containing 40 μ M WT or G93A-SOD1 in absence or
14 presence of 25 mM DTT were incubated at 37 °C with agitation at 300 rpm. To monitor the SOD1
15 aggregation, 40 μ L of the reaction mixtures were mixed with 260 μ L of 120 μ M ThT at 4 h intervals and
16 the fluorescence was measured using a F-4500 Fluorescence Spectrophotometer at $\lambda_{\text{ex}} = 450$ nm (slit
17 width = 5 nm) and $\lambda_{\text{em}} = 485$ nm (slit width = 10 nm).

18
19 *Electron Microscopy.* Ten- μ L aliquots from the ThT reactions in the second format were applied to glow-
20 discharged, carbon-coated Formvar grids (Electron Microscopy Sciences, FCF400-CU) as described
21 previously^{35, 36} and incubated for 2 min. The solution was wicked off gently using Whatman, grade-2
22 filter paper. The proteins were stained with 10 μ L of 1% uranyl acetate (Ted Pella, Inc.) for 2 min and
23 wicked dry using filter paper. The grid was washed 3 times with deionized water at 2-min intervals, the
24 solutions were wicked off, and the grids were air-dried. The samples were examined using a JEOL 1200
25 electron microscope operating at 80 keV.

26
27 *Dot Blot.* Two- μ L aliquots of each reaction mixture were taken every 4 h (WT and G93A-SOD1) or every
28 8 h (E100K-SOD1) during the aggregation reactions and loaded onto nitrocellulose membranes. Two
29 independent experiments were conducted with 6 replicates each of reducing (all three variants) and non-
30 reducing (WT and G93A-SOD1 only) conditions. The metallated proteins (WT and G93A-SOD1 only)
31 were probed without incubation as a control in separate dot-blot. The membranes were incubated in ~10
32 mL of blocking buffer containing 5% non-fat, dry-milk powder in 20 mM Tris HCl, 150 mM NaCl, 0.1%
33 Tween-20, pH 7.4 (TBST), with mild agitation for 1 hour at room temperature or overnight at 4 °C. Then,
34 the membranes were incubated with 10 mL solutions of each primary antibody in blocking buffer
35 overnight at 4 °C. The following commercial antibodies and dilutions were used: Ab16831 (Abcam,
36 1:2,000), 2770 (Cell Signaling Technology, 1:1,000), B8H10 (MédiMabs, 1:500), and C4F6 (MédiMabs,
37 1:500). Additionally, the following new anti-misfolded SOD1 antibodies and dilutions were used: AMF7-
38 63 (1:500), 10C12 (1:500), and 3H1 (1:500).

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40 The membranes were washed with blocking buffer twice for 5 min and then incubated with 10 mL of
41 horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology,
42 1:2,000 dilution) or horse anti-mouse secondary antibody (Cell Signaling Technology, 1:2,000), as
43 appropriate, for 2 h at room temperature with mild agitation and washed twice with 10 mL of TBST for
44 10 min. Finally, the dots were visualized using a HyGlo™ Quick Spray chemiluminescence kit (Denville
45 Scientific). Images were captured using a Syngene multi-application gel imaging system PXi and
46 quantified using ImageJ.³⁷ After image visualization, the membranes were rehydrated and stripped of
47 antibodies using a Blot Restore Membrane Rejuvenation Kit (Millipore Sigma). The membranes then
48 were re-probed with the next antibody. This process was repeated to analyze multiple antibodies using the
49 same membranes.

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51 *Native-PAGE/Western blot.* Two- μ g total protein per sample from each time point were fractionated using
52 NuPage 4-16% Bis-Tris gels in triplicate and the proteins were transferred to 0.2- μ m pore size
53 nitrocellulose membranes. Membranes were probed using the seven antibodies in the following dilutions:
54 Ab16831 – 1:2,000, 2770 – 1:1,000, B8H10 – 1:500, C4F6 – 1:500, AMF7-63 – 1:500, 3H1 – 1:500,
55 10C12 – 1:500. HRP-conjugated secondary anti-mouse or anti-rabbit antibodies were used at 1:10,000
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3 and 1:2,000 dilutions, respectively, and bands were visualized using HyGLO Quick Spray
4 chemiluminescent detection reagent (Denville, #E2400) and imaged using a Syngene PXi digital imager
5 (Syngene, Frederick, MD). After imaging, the antibodies were stripped using RestoreTM Plus Western blot
6 stripping buffer (Thermo Scientific) and then the blots were re-probed using a different order of
7 antibodies for each replicate.
8

9 *LC-MS*. A 3- μ L solution of WT or G93A-SOD1 was incubated in the presence of 0.25 M DTT for 10 min
10 at room temperature, mixed with 7 μ L of formic acid and fractionated into a ThermoFisher LTQ XL
11 mass-spectrometer via a 2-mm SW2000 capillary column using a 4:4:1 mixture of
12 chloroform:methanol:1% formic acid. Mass spectra were recorded in a positive-ion mode. Spectra were
13 deconvoluted using MagTran.
14

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Antibody	Raised in	Antigen	Type	Expected reactivity/specificity
Ab16831 (Abcam)	Rabbit	Total human WT SOD1	pAb*	Total SOD1
2770 (Cell Signalling Technology)	Rabbit	Human SOD1 N-terminal epitope	pAb	Total SOD1
B8H10 (MédiMabs)	Mouse	Human G93A SOD1	mAb*	Misfolded SOD1
C4F6 (MédiMabs)	Mouse	Human G93A SOD1	mAb	Misfolded SOD1
AMF7-63	Rabbit	The electrostatic loop D ¹²⁵ DLGKGGNEESTKTGNAG ¹⁴²	mAb	High affinity for misfolded SOD1
3H1	Mouse	The electrostatic loop D ¹²⁵ DLGKGGNEESTKTGNAG ¹⁴²	mAb	1,000-fold lower affinity than AMF7-63
10C12	Mouse	SOD1-exposed dimer interface (SEDI) ¹³ with Cys146 replaced by cysteine acid	mAb	Oxidized misfolded SOD1 monomers/oligomers/aggregates
* mAb = monoclonal antibody. pAb = polyclonal antibody.				

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Table 2: Reactivity of different antibodies with WT and G93A						
Antibody reactivity with:	Metallated Form reactivity¹	Reactivity at t = 0 h²	Reactivity at t = 4 h²	Maximal reactivity time (Figure 4/Figure 5)	Maximal reactivity³	Reactivity at t = 40 h^{2,4}
WT-SOD1						
Ab16831	R	1,086 ± 990	1,651 ± 1,595	8/12	2,373 ± 1,359	1,288 ± 1,228
2770	R	1,596 ± 727	1,980 ± 848	4/4 ⁵	1,980 ± 848	1,336 ± 1,195
B8H10	R	1,384 ± 367	1,973 ± 535	12/12 ⁵	2,032 ± 699	868 ± 843
C4F6	U	0 ± 0	0 ± 0	-	0 ± 0	-
AMF-7-63	R	993 ± 293	1,672 ± 576	12/40	2,316 ± 732	1,985 ± 1,408
3H1	U	547 ± 108	1,188 ± 226	12/24	1,834 ± 719	1,286 ± 1,022
10C12	U	1,603 ± 438	4,171 ± 1,180	8/12	4,929 ± 947	2,502 ± 1,804
G93A-SOD1						
Ab16831	R	2,238 ± 909	3,313 ± 817	12/16	4,785 ± 1,310	550 ± 171
2770	R	3,472 ± 1,189	4,381 ± 1,295	4/16	5,182 ± 708	431 ± 347
B8H10	R	2,570 ± 845	3,166 ± 476	12/16	5,285 ± 1,636	797 ± 301
C4F6	U	1,933 ± 333	1,933 ± 166	4/8 ⁵	2,029 ± 319	1,174 ± 664
AMF-7-63	R	1,779 ± 533	2,740 ± 819	12/16	5,164 ± 656	1,664 ± 997
3H1	U	585 ± 177	1,027 ± 259	12/20	3,418 ± 1,459	1,829 ± 1,920
10C12	U	3,220 ± 803	4,099 ± 701	4/8	4,919 ± 393	478 ± 248
¹ The metallated form was analyzed separately from the apo form and therefore the two cannot be compared quantitatively. Therefore, we only indicate if the metallated form was reactive (R) or unreactive (U). ² Values are average of the densitometry values shown in Figures 4C,D and 5C,D. ³ The reactivity at the maximal time each in Figures 4 and 5 was averaged. ⁴ The reactivity in fast reactions (Figure 4) was substantially lower than that of slow reactions (Figure 5). ⁵ The antibody showed relatively little variation with time.						

Figure legends:

Figure 1. Temporal change in β -sheet content of WT or G93A-SOD1 measured using ThT fluorescence. Forty μ M of WT (A, C) or G93A- (B, D) SOD1 were incubated at 37 °C with fast agitation in the absence (A, B) or presence (C, D) of 25 mM DTT and the change in ThT fluorescence was monitored in a plate reader for 40 h. To facilitate comparison, all the reactions are shown with the same scale in the y-axis. In panels A and B, the insets show the small changes in fluorescence using adjusted y-axis scales. In panels C and D, insets show one reaction with the lag phase (L), first small increase (I1), first plateau (P1), second large increase (I2), and final plateau (P2) indicated.

Figure 2. Morphological changes in WT and G93A-SOD1 during incubation. Aliquots from aggregation reactions were taken for morphological examination by EM. Representative images are shown for the WT and G93A-SOD1 isoforms. The scale bars denote 100 μ m.

Figure 3. Antibody reactivity for WT and G93A-SOD1 under non-reducing conditions. Aliquots from the same aggregation reactions shown in Figure 1 were taken every 4 h and dot-blotted on nitrocellulose membranes. At the end of the experiment, each membrane was blocked and then probed, stripped, and re-probed with the seven antibodies. The order of the antibodies was changed in different replicates to avoid bias due to loss of protein during strip and re-probe cycles. A, B) Representative dot blots of the apo forms of WT (A) and G93A- (B) SOD1 incubated under non-reducing conditions at different time points. The metallated form of each protein is shown on the left (indicated by M) as a control. C, D) Averaged densitometric analysis of two independent experiments, each with 6 replicates (n = 12) of WT (C) and G93A- (D) SOD1. The lines are added to help guide the eye but do not imply that the points should be connected. Filled symbols represent cases in which the change in antibody binding during the reaction was found to be statistically significant, whereas empty symbols represent insignificant changes (1-way ANOVA). E, F) Representative ThT fluorescence curves from the same reactions of WT (E) and G93A- (F) SOD1.

Figure 4. Antibody reactivity for WT and G93A-SOD1 under reducing conditions in reactions with short “pause” (P1) periods. Aliquots from the same aggregation reactions shown in Figure 1 were taken every 4 h and dot-blotted on nitrocellulose membranes. At the end of the experiment, each membrane was blocked and then probed, stripped, and re-probed with the 7 different antibodies. The order of the antibodies was changed in different experiments to avoid bias due to loss of protein during strip and re-probe cycles. A, B) Representative dot blots of WT (A) and G93A- (B) SOD1 reactions in which the initial plateau phase, P1, was relatively short, i.e., ≤ 10 h for WT SOD1 and ≤ 7 h for G93A-SOD1, incubated under reducing conditions at different time points. C, D) Averaged values from densitometric analysis of two independent experiments, including a total of 9 replicates of WT (C) and 6 replicates of G93A- (D) SOD1. The lines are added to help guide the eye but do not imply that the points should be connected. Filled symbols represent cases in which the change in antibody binding during the reaction was found to be statistically significant, whereas empty symbols represent insignificant changes (1-way ANOVA). E, F) Representative ThT fluorescence curves from the same reactions of WT (E) and G93A- (F) SOD1.

Figure 5. Antibody reactivity for WT and G93A-SOD1 under reducing conditions in reactions with long “pause” (P1) periods. Aliquots from the same aggregation reactions shown in Figure 1 were taken every 4 h and dot-blotted on nitrocellulose membranes. At the end of the experiment, each membrane was blocked and then probed, stripped, and re-probed with the 7 different antibodies. The order of the antibodies was changed in different experiments to avoid bias due to loss of protein during strip and re-probe cycles. A, B) Representative dot blots of WT (A) and G93A- (B) SOD1 reactions in which the initial plateau phase, P1, was relatively long, i.e., > 10 h for WT SOD1 and > 7 h for G93A-SOD1, incubated under reducing conditions at different time points. C, D) Averaged values from densitometric analysis of two independent experiments, including a total of 3 replicates of WT (C) and 6 replicates of G93A- (D) SOD1. The lines are added to help guide the eye but do not imply that the points should be

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3 connected. Filled symbols represent cases in which the change in antibody binding during the reaction
4 was found to be statistically significant, whereas empty symbols represent insignificant changes (1-way
5 ANOVA). E, F) Representative ThT fluorescence curves from the same reactions of WT (E) and G93A-
6 (F) SOD1.
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8 **Figure 6. Mass-spectrometry characterization of WT and G93A-SOD1.** Each protein was incubated
9 in the presence of 0.25 M DTT for 10 min at room temperature and then subjected to LC-MS analysis. A)
10 Recorded (top) and deconvoluted (bottom) spectra of WT SOD1. B) Recorded (top) and deconvoluted
11 (bottom) spectra of G93A-SOD1. The fragmentation type (*A*, *B*, or *C*) and charge of each fragment is
12 indicated in red and the observed mass in black.
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14 **Figure 7. Native-PAGE/Western blot analysis of WT and G93A-SOD1 during aggregation.** Forty
15 μM of WT (A, C, E) or G93A- (B, D, F) SOD1 were incubated at 37 °C with fast agitation in the presence
16 (C, of 25 mM DTT. Aliquots were taken every 4 h, flash frozen, and stored at -80 °C. At the end of the
17 reactions, the aliquots were fractionated by native PAGE and the proteins were transferred to
18 nitrocellulose membranes and probed with each antibody. Only Ab16831 (A,B), 2770 (C,D), and B8H10
19 yielded sufficient signal of signal-to-noise ratio and therefore the other antibodies are not shown. NR =
20 non-reduced. Positions of molecular-weight markers are shown on the left of each membrane.
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22 **Figure 8. ThT fluorescence and antibody reactivity for E100K-SOD1.** Forty μM of E100K-SOD1
23 were incubated at 37 °C with fast agitation in the presence of 25 mM DTT. A) The change in ThT
24 fluorescence was monitored in a plate reader for 40 h. B) Representative dot blots with each antibody. C)
25 Averaged values from densitometric analysis of two independent experiments including a total of 4
26 replicates. The lines are added to help guide the eye but do not imply that the points should be connected.
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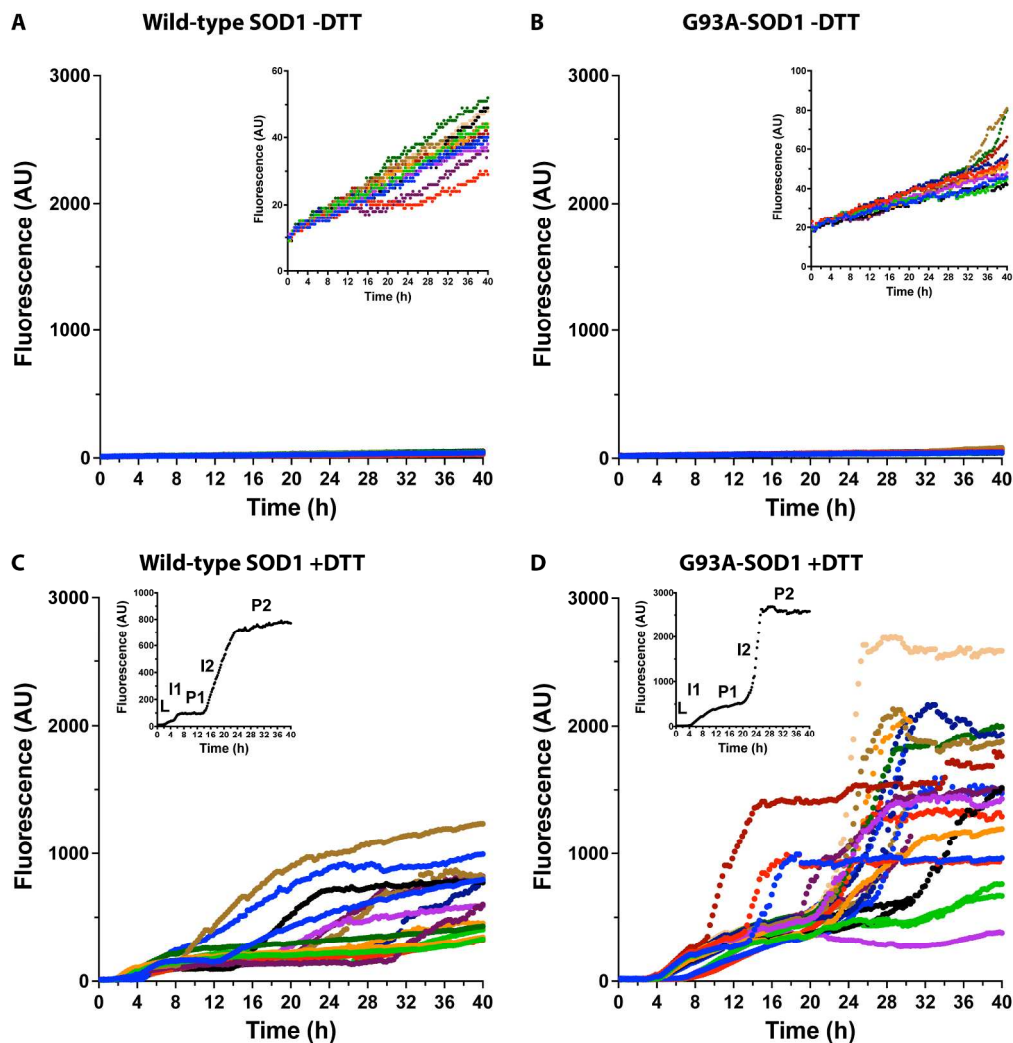
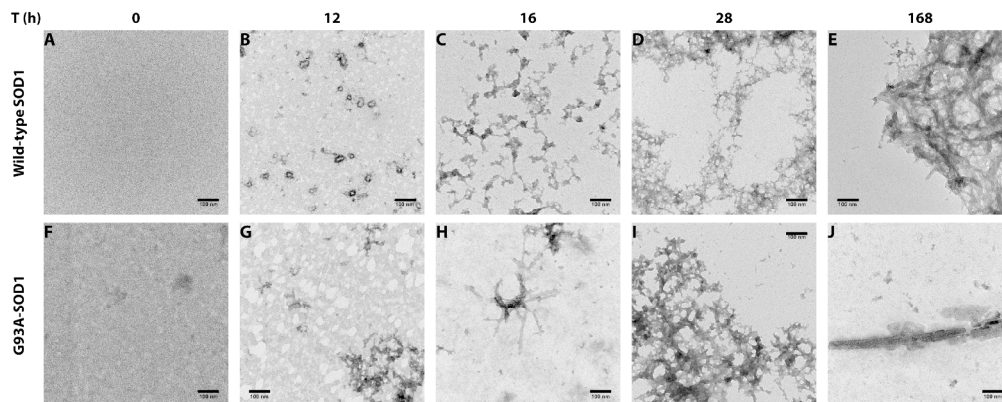


Figure 1. Temporal change in β -sheet content of WT or G93A-SOD1 measured using ThT fluorescence. Forty μ M of WT (A, C) or G93A- (B, D) SOD1 were incubated at 37 $^{\circ}$ C with fast agitation in the absence (A, B) or presence (C, D) of 25 mM DTT and the change in ThT fluorescence was monitored in a plate reader for 40 h. To facilitate comparison, all the reactions are shown with the same scale in the y-axis. In panels A and B, the insets show the small changes in fluorescence using adjusted y-axis scales. In panels C and D, insets show one reaction with the lag phase (L), first small increase (I1), first plateau (P1), second large increase (I2), and final plateau (P2) indicated.

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Figure 2. Morphological changes in WT and G93A-SOD1 during incubation. Aliquots from aggregation reactions were taken for morphological examination by EM. Representative images are shown for the WT and G93A-SOD1 isoforms. The scale bars denote 100 μ m.

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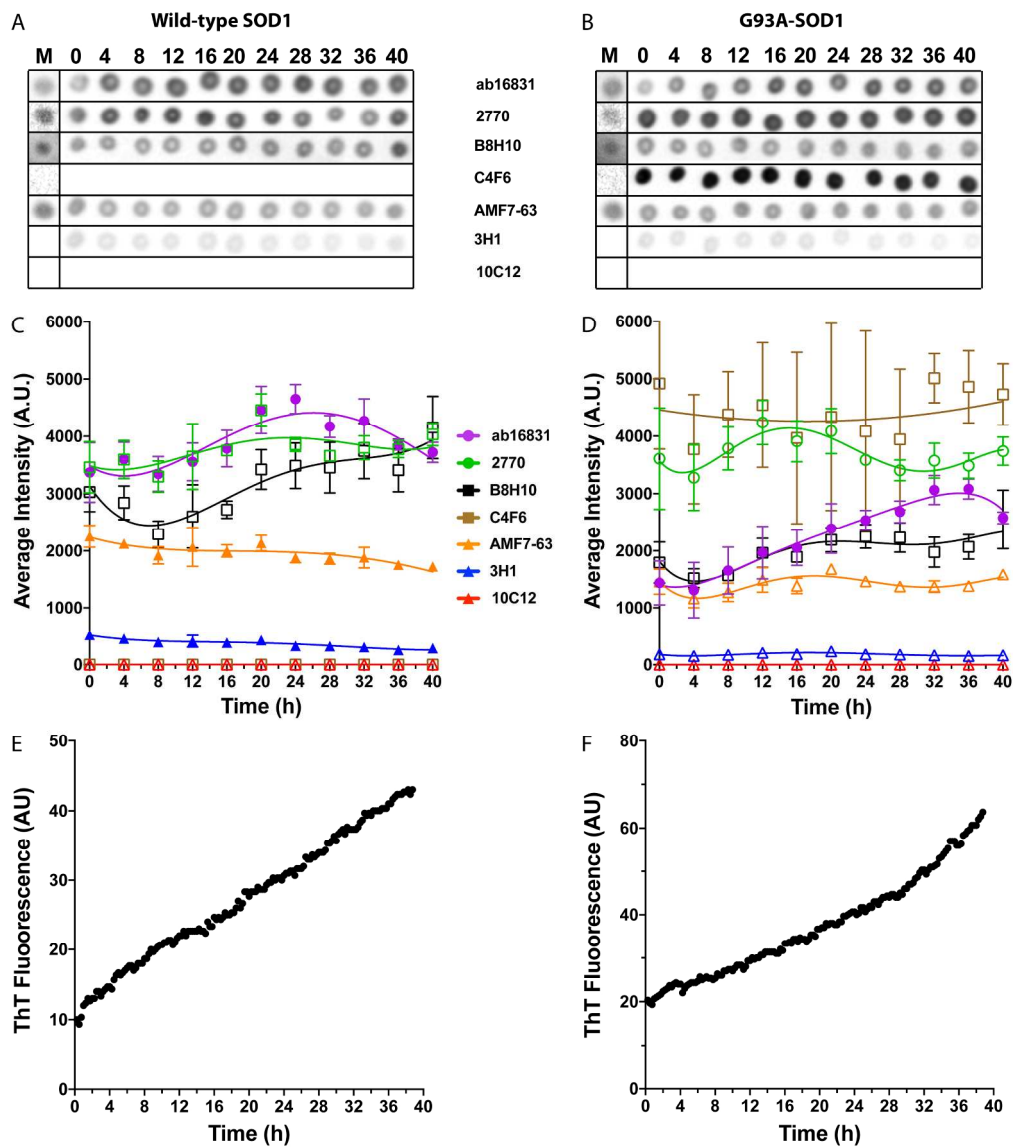


Figure 3. Antibody reactivity for WT and G93A-SOD1 under non-reducing conditions. Aliquots from the same aggregation reactions shown in Figure 1 were taken every 4 h and dot-blotted on nitrocellulose membranes. At the end of the experiment, each membrane was blocked and then probed, stripped, and re-probed with the seven antibodies. The order of the antibodies was changed in different replicates to avoid bias due to loss of protein during strip and re-probe cycles. A, B) Representative dot blots of the apo forms of WT (A) and G93A- (B) SOD1 incubated under non-reducing conditions at different time points. The metallated form of each protein is shown on the left (indicated by M) as a control. C, D) Averaged densitometric analysis of two independent experiments, each with 6 replicates ($n = 12$) of WT (C) and G93A- (D) SOD1. The lines are added to help guide the eye but do not imply that the points should be connected. Filled symbols represent cases in which the change in antibody binding during the reaction was found to be statistically significant, whereas empty symbols represent insignificant changes (1-way ANOVA). E, F) Representative ThT fluorescence curves from the same reactions of WT (E) and G93A- (F) SOD1.

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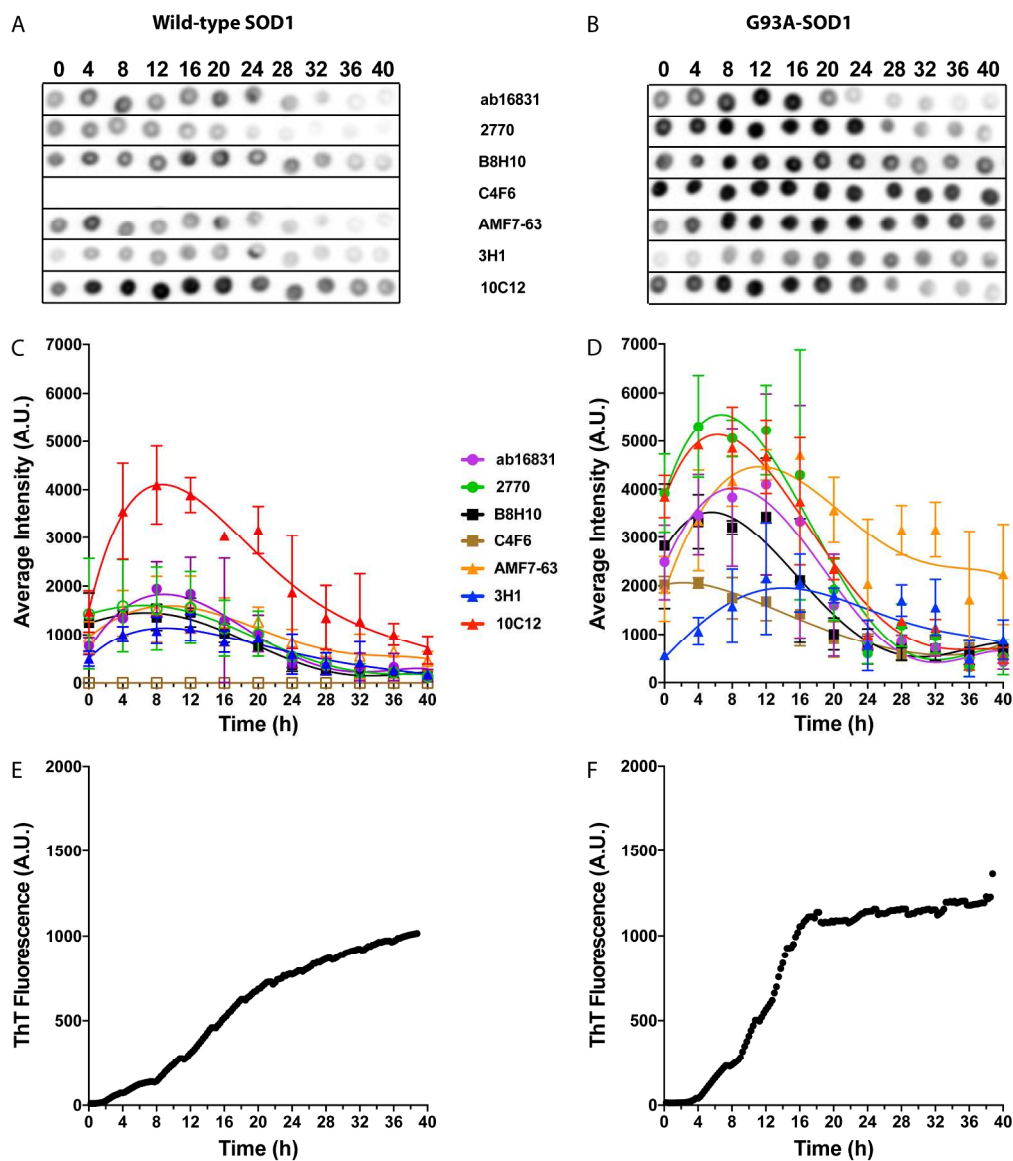


Figure 4. Antibody reactivity for WT and G93A-SOD1 under reducing conditions in reactions with short “pause” (P1) periods. Aliquots from the same aggregation reactions shown in Figure 1 were taken every 4 h and dot-blotted on nitrocellulose membranes. At the end of the experiment, each membrane was blocked and then probed, stripped, and re-probed with the 7 different antibodies. The order of the antibodies was changed in different experiments to avoid bias due to loss of protein during strip and re-probe cycles. A, B) Representative dot blots of WT (A) and G93A- (B) SOD1 reactions in which the initial plateau phase, P1, was relatively short, i.e., ≤ 10 h for WT SOD1 and ≤ 7 h for G93A-SOD1, incubated under reducing conditions at different time points. C, D) Averaged values from densitometric analysis of two independent experiments, including a total of 9 replicates of WT (C) and 6 replicates of G93A- (D) SOD1. The lines are added to help guide the eye but do not imply that the points should be connected. Filled symbols represent cases in which the change in antibody binding during the reaction was found to be statistically significant, whereas empty symbols represent insignificant changes (1-way ANOVA). E, F) Representative ThT fluorescence curves from the same reactions of WT (E) and G93A- (F) SOD1.

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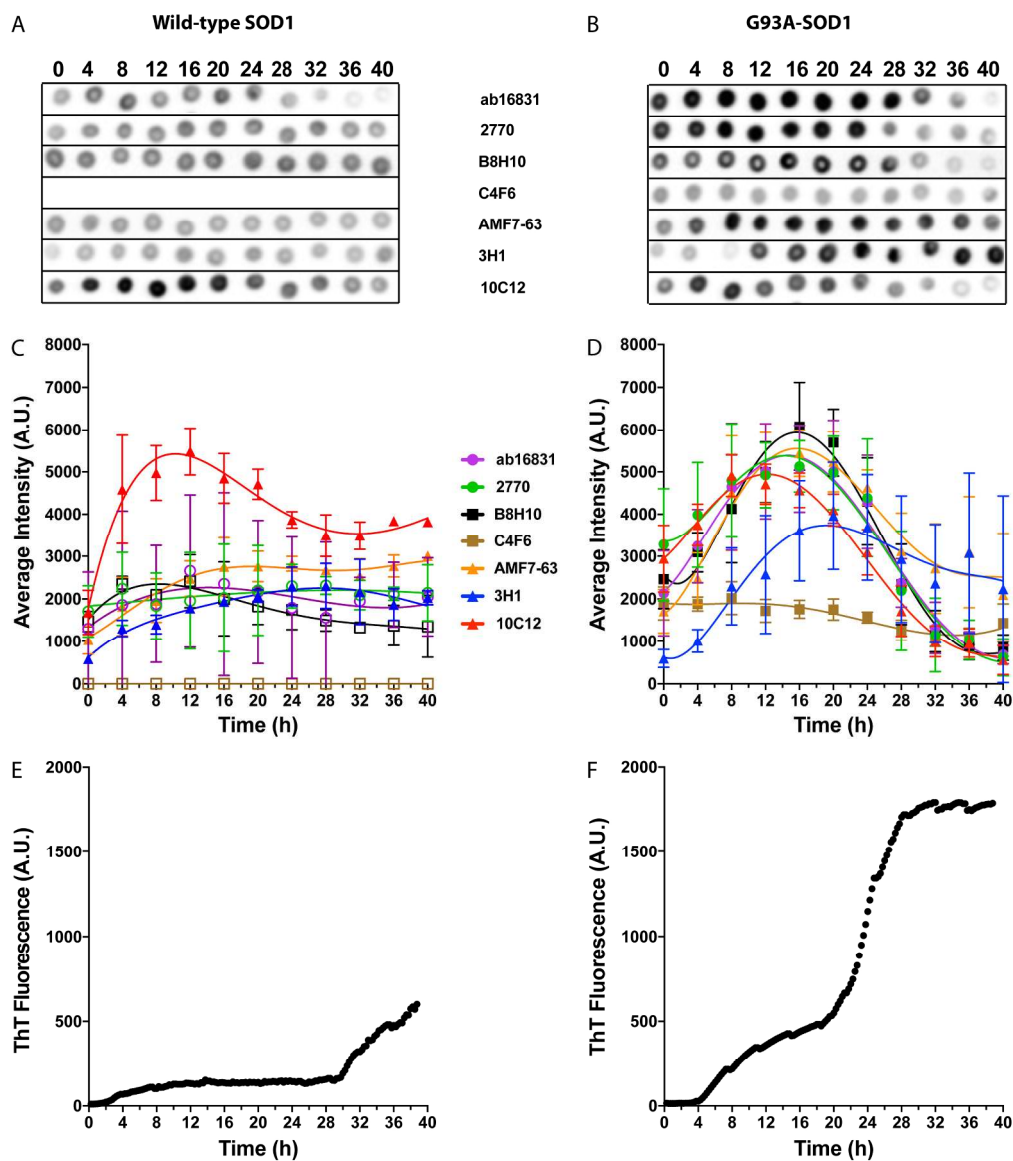


Figure 5. Antibody reactivity for WT and G93A-SOD1 under reducing conditions in reactions with long “pause” (P1) periods. Aliquots from the same aggregation reactions shown in Figure 1 were taken every 4 h and dot-blotted on nitrocellulose membranes. At the end of the experiment, each membrane was blocked and then probed, stripped, and re-probed with the 7 different antibodies. The order of the antibodies was changed in different experiments to avoid bias due to loss of protein during strip and re-probe cycles. A, B) Representative dot blots of WT (A) and G93A- (B) SOD1 reactions in which the initial plateau phase, P1, was relatively long, i.e., > 10 h for WT SOD1 and > 7 h for G93A-SOD1, incubated under reducing conditions at different time points. C, D) Averaged values from densitometric analysis of two independent experiments, including a total of 3 replicates of WT (C) and 6 replicates of G93A- (D) SOD1. The lines are added to help guide the eye but do not imply that the points should be connected. Filled symbols represent cases in which the change in antibody binding during the reaction was found to be statistically significant, whereas empty symbols represent insignificant changes (1-way ANOVA). E, F) Representative ThT fluorescence curves from the same reactions of WT (E) and G93A- (F) SOD1.

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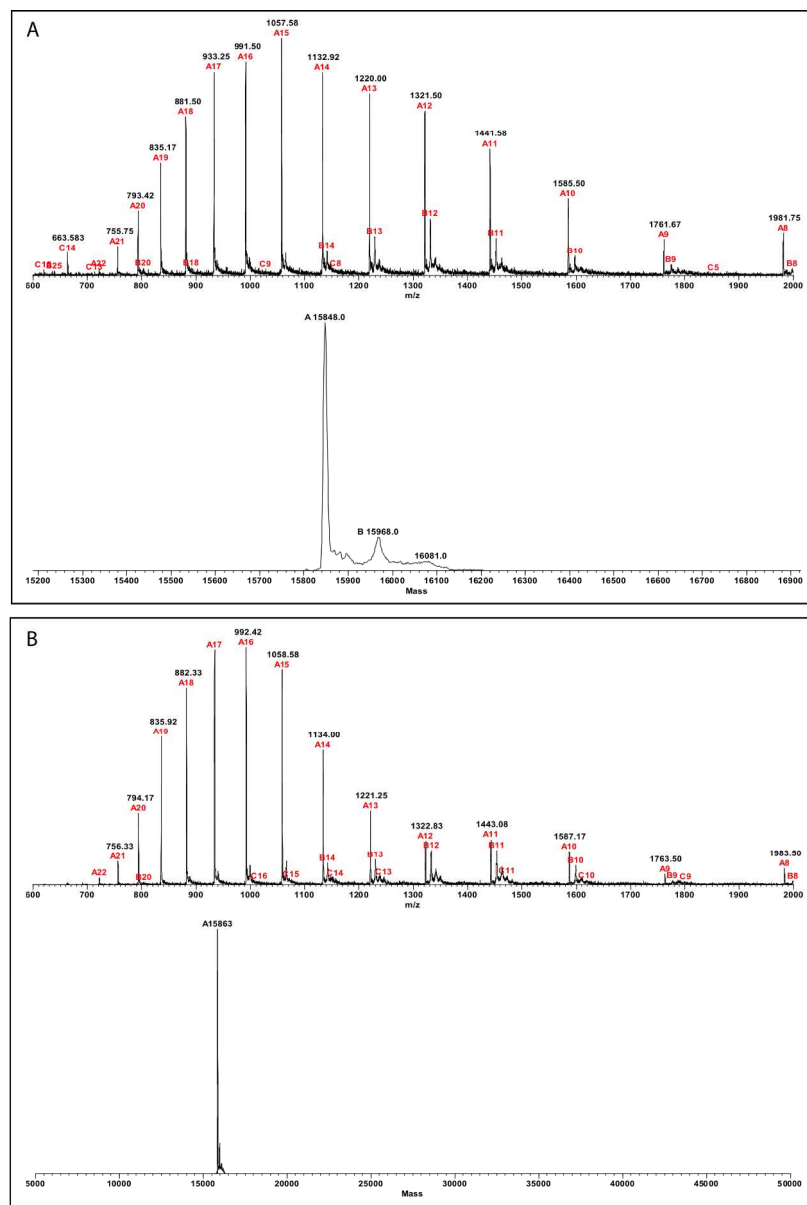


Figure 6. Mass-spectrometry characterization of WT and G93A-SOD1. Each protein was incubated in the presence of 0.25 M DTT for 10 min at room temperature and then subjected to LC-MS analysis. A) Recorded (top) and deconvoluted (bottom) spectra of WT SOD1. B) Recorded (top) and deconvoluted (bottom) spectra of G93A-SOD1. The fragmentation type (A, B, or C) and charge of each fragment is indicated in red and the observed mass in black.

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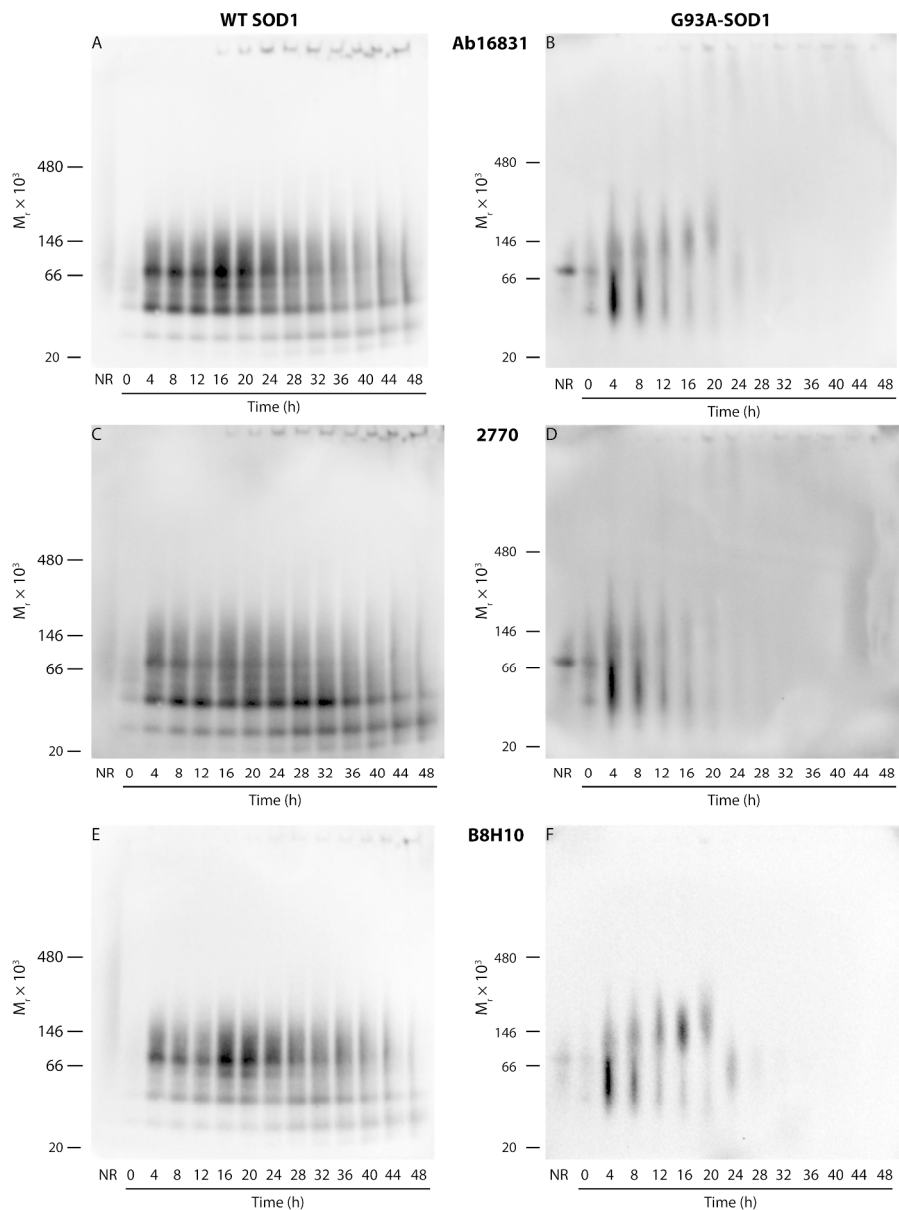


Figure 7. Native-PAGE/Western blot analysis of WT and G93A-SOD1 during aggregation. Forty μM of WT (A, C, E) or G93A- (B, D, F) SOD1 were incubated at 37 $^{\circ}\text{C}$ with fast agitation in the presence (C, of 25 mM DTT). Aliquots were taken every 4 h, flash frozen, and stored at -80 $^{\circ}\text{C}$. At the end of the reactions, the aliquots were fractionated by native PAGE and the proteins were transferred to nitrocellulose membranes and probed with each antibody. Only Ab16831 (A,B), 2770 (C,D), and B8H10 yielded sufficient signal of signal-to-noise ratio and therefore the other antibodies are not shown. NR = non-reduced. Positions of molecular-weight markers are shown on the left of each membrane.

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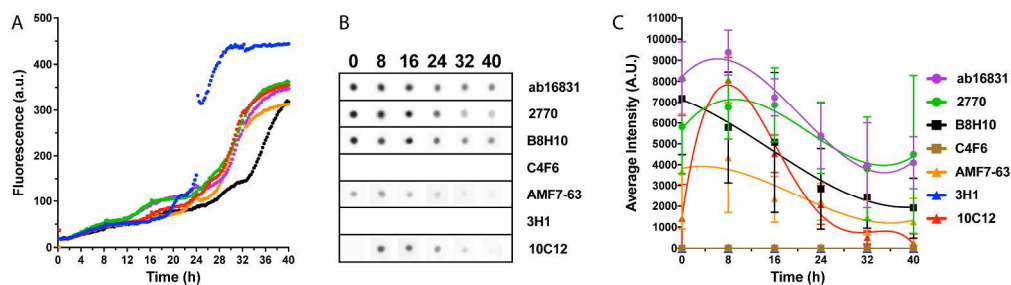
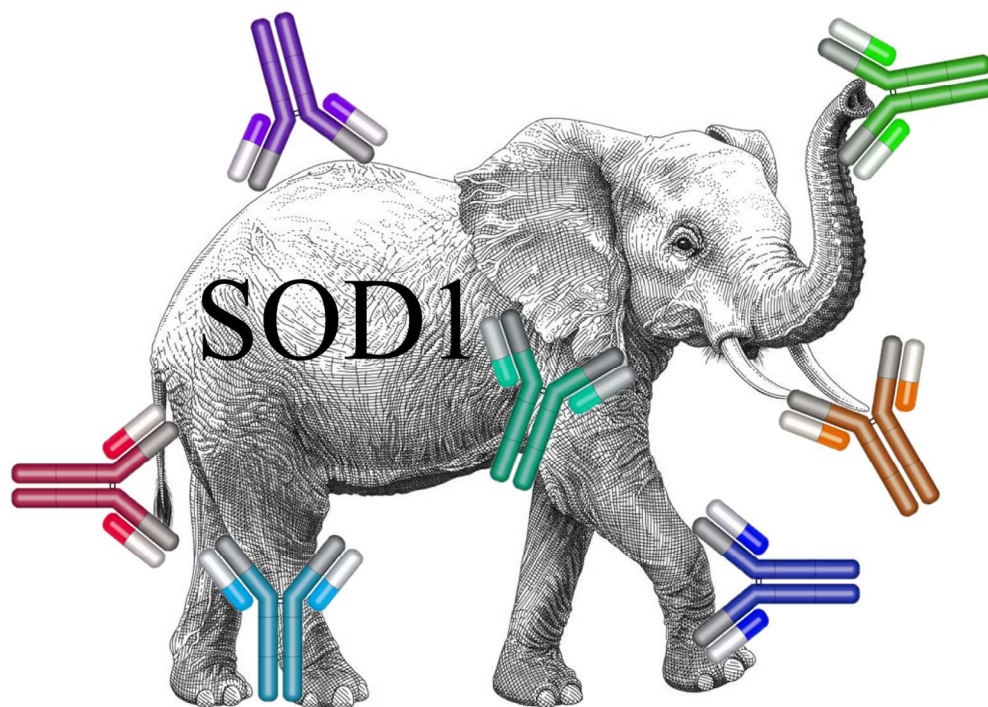


Figure 8. ThT fluorescence and antibody reactivity for E100K-SOD1. Forty μM of E100K-SOD1 were incubated at 37 $^{\circ}\text{C}$ with fast agitation in the presence of 25 mM DTT. A) The change in ThT fluorescence was monitored in a plate reader for 40 h. B) Representative dot blots with each antibody. C) Averaged values from densitometric analysis of two independent experiments including a total of 4 replicates. The lines are added to help guide the eye but do not imply that the points should be connected.

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TOC Graphic

146x110mm (300 x 300 DPI)