Published in final edited form as:

Biochem Eng J. 2014 October 15; 91: 196–203. doi:10.1016/j.bej.2014.08.013.

Suppressing mutation-induced protein aggregation in mammalian cells by mutating residues significantly displaced upon the original mutation

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Abstract

Mutations introduced to wild-type proteins naturally, or intentionally via protein engineering, often lead to protein aggregation. In particular, protein aggregation within mammalian cells has significant implications in the disease pathology and biologics production; making protein aggregation modulation within mammalian cells a very important engineering topic. Previously, we showed that the semi-rational design approach can be used to reduce the intracellular aggregation of a protein by recovering the conformational stability that was lowered by the mutation. However, this approach has limited utility when no rational design approach to enhance conformational stability is readily available. In order to overcome this limitation, we investigated whether the modification of residues significantly displaced upon the original mutation is an effective way to reduce protein aggregation in mammalian cells. As a model system, human copper, zinc superoxide dismutase mutant containing glycine to alanine mutation at position 93 (SOD1^{G93A}) was used. A panel of mutations was introduced into residues substantially displaced upon the G93A mutation. By using cell-based aggregation assays, we identified several novel variants of SOD1^{G93A} with reduced aggregation propensity within mammalian cells. Our findings successfully demonstrate that the aggregation of a mutant protein can be suppressed by mutating the residues significantly displaced upon the original mutation.

Keywords

protein aggregation; mammalian cells; mutation; protein engineering; superoxide dismutase

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SUPPORTING INFORMATION

Supporting information is available online.

The authors have no conflict of interest.

1. INTRODUCTION

Protein misfolding and aggregation caused by natural mutations have been implicated in many human diseases [1]. Therefore, suppressing protein aggregation is considered an effective strategy to treat numerous protein aggregation-associated diseases [2–10]. Furthermore, many biologics approved for human use have been produced using mammalian cells, such as Chinese hamster ovary cells. From 2006 to 2010, approximately 58 biologics gained FDA approval, 32 of which were produced solely in mammalian cells, intimating the increased importance of understanding protein production in mammalian cells [11]. In particular, non-native aggregation of proteins presents a significant challenge at all stages of biologics production [12–16]. Although strategies were developed to modulate protein aggregation *in vitro* in bacterial cells [17–20], it is not straightforward to apply them to mammalian cells due to differences in protein synthesis and folding machinery [21]. Therefore, the screening of protein variants in mammalian cells is beneficial to identify protein variants with a reduced aggregation propensity in mammalian cells.

Recently it was demonstrated that soluble intrabodies can be obtained by screening the intrabody libraries in mammalian cells [22]. Complementary to high-throughput screening, we used the semi-rational design approach to arrest the aggregation of a human copper, zinc superoxide dismutase mutant containing alanine to valine mutation at position 4 (SOD1^{A4V}) inside mammalian cells by enhancing the thermodynamic conformational stability [23]. Although wild-type SOD1 (SOD1^{WT}) is very stable and does not form intracellular aggregates, the A4V mutation greatly increases the aggregation-propensity. In our previous studies, the mutation of a bulky side chain of phenylalanine at position 20 (F20), in direct contact with the side chain of valine at position 4 (V4), into smaller ones successfully eliminated the steric hindrance between the side chains of F20 and V4 resulting in the enhanced thermodynamic conformational stability and reduced protein aggregation [23]. This approach is applicable in cases where protein destabilization is caused by repulsive interactions between side chains, but not to cases where the repulsive interactions between a side chain of mutated residue and backbone protein induces protein destabilization. To our knowledge, no rational design approach to redesign protein backbones to eliminate such destabilizing effects has been reported. Therefore, the development of alternative strategies is required.

It is noteworthy that the destabilizing effects of the original mutation often cause structural perturbations resulting in the displacement of several residues within close proximity of the mutated residue. Therefore, we hypothesized that the mutation of residues significantly displaced upon the original mutation can reduce the destabilizing effects leading to reduced intracellular aggregation in mammalian cells. As a model protein, we chose a SOD1 variant containing glycine to alanine mutation at position 93 (SOD1^{G93A}) associated with the familial form of amyotrophic lateral sclerosis (fALS), a fatal muscular neurodegenerative disease. Wild-type SOD1 and its variants are attractive candidates to develop techniques to suppress protein aggregation in mammalian cells. We believe that the methods and schemes established using a model protein would be applicable to therapeutic proteins (cytokines and antibodies) for several reasons. First, SOD1 is human protein and has biological activities. Second, similar to antibodies, subtle perturbations of folded structure lead to aggregation,

which is evidenced by many mutations in SOD1 causing aggregation. Second, similar to antibodies and cytokines, intramolecular disulfide bonds are critical for correct folding. Third, they are easily expressed in mammalian cells. Fourth, crystal structures of many SOD1 variants are available to facilitate structural analysis. In particular, SOD1^{G93A} is suitable for our studies on suppressing intracellular aggregation without any apparent steric hindrance, because a side chain of the mutated residue (Ala93) does not contact with any side chain of neighboring residues but contacts with neighboring backbones in the crystal structure of SOD1^{G93A} (PDB ID:2KWO) [24]. Furthermore, SOD1^{G93A} has the high aggregation propensity inside mammalian cells [24–26].

2. MATERIAL AND METHODS

2.1 Materials

The HRP-conjugated anti-rabbit antibody was obtained from Invitrogen (Carlsbad, CA). The anti-SOD1 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). HEK293T and NSC-34 cells were obtained from Invitrogen and CELLutions Biosystems (Burlington, Ontario, CA), respectively. Primers used for construction of expression vectors were obtained from Invitrogen. Gibco Certified Fetal Bovine Serum (FBS) was obtained from Invitrogen. All other chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

2.2 Methods

2.2.1 Construction of Expression Vectors—The pEGFP-N3-SOD1^{WT} plasmid was kindly provided by Dr. Haining Zhu (University of Kentucky). The mutations were introduced into the pEGFP-N3-SOD1^{WT} using the QuikChange II site-directed mutagenesis kit (Stratagene). The primer pairs for each mutant are as follows: 5'-GACTGCTGACAAAGATGCTGTGGCCGATGTGTC-3' and 5'-GACACATCGGCCACAGCATCTTTGTCAGCAGTC-3' for G93A; 5'-GCCTTCAGTCAGTCCCCTCAATGCTTCCCCACACCTTCAC-3 and 5'-GTGAAGGTGTGGGGAAGCATTGAGGGACTGACTGAAGGC-3' for K36E; 5'-AGTGAAGGTGTGGGGAAGCATTTTAGGACTGACTGAAGG-3' and 5'-CCTTCAGTCAGTCCTAAATGCTTCCCCACACCTTCACT-3' for K36L; 5'-GGCAATGTGACTGCTGACGCAGATGCTGTGGCCGATGTGTCTAT-3' and 5'-ATAGACACATCGGCCACAGCATCTGCGTCAGCAGTCACATTGCC-3' for K91A/G93A; 5'-GGCAATGTGACTGCTGACGATGATGCTGTGGCCGATGTGTCTAT-3' and 5'-ATAGACACATCGGCCACAGCATCATCGTCAGCAGTCACATTGCC-3' for K91D/G93A; 5'-ATAGACACATCGGCCACAGCATCCTCGTCAGCAGTCACATTGCC-3' and 5'-GGCAATGTGACTGCTGACGAGGATGCTGTGGCCGATGTGTCTAT-3' for K91E/G93A; 5'-GGCAATGTGACTGCTGACAGAGATGCTGTGGCCGAT-3' and 5'-ATCGGCCACAGCATCTCTGTCAGCAGTCACATTGCC-3' for K91R/G93A; 5'-CATTAAAGGACTGACTGCAGGCCTGCATGGATTCC-3 and 5'-GGAATCCATGCAGGCCTGCAGTCAGTCCTTTAATG-3' for E40A; 5'-GAATCCATGCAGGCCTTTAGTCAGTCCTTTAATGC-3' and 5'-GCATTAAAGGACTGACTAAAGGCCTGCATGGATTCC-3' for E40K; 5'-GGAAGCATTAAAGGACTGACTGATGGCCTGCATGGA-3' and 5'-

TCCATGCAGGCCATCAGTCAGTCCTTTAATGCTTCC -3' for E40D; 5'-CCATGCAGGCCTCTAGTCAGTCCTTTAATGCTTCCCC -3' and 5'-GGGGAAGCATTAAAGGACTGACTAGAGGCCTGCATGG-3' for E40R. The sequences of the mutations were confirmed using DNA sequencing.

2.2.2 Computational Analysis of Protein Structure—The computational analysis of SOD1 conformational stability was performed using the molecular modeling program RosettaDesign version 3.4 [27]. The core energy function of RosettaDesign is a linear sum of a 6–12 Lennard-Jones potential, the Lazaridis-Karplus implicit solvation model, an empirical hydrogen bonding potential, backbone-dependent rotamer probabilities, a knowledge-based electrostatics energy potential, amino acid probabilities based on particular regions of ϕ/ψ space, and a unique reference energy for each amino acid [27]. For a given crystal structure, RosettaDesign uses simulated annealing to scan through a large number of rotamers to minimize the energy score with Monte Carlo optimization [28].

A fixed backbone protein design protocol was used in this study to identify the key interactions responsible for SOD1^{G93A} destabilization. The holo-form of SOD1^{WT} crystal structure (PDB ID: 2C9V) was used [29, 30]. A homodimeric holo-form of SOD1^{G93A-WT} structure was constructed from the SOD1^{WT} structure by repacking all protein side chains except those chelated to metal ions (H46, H63, H71, H80, D83 and H120) to adopt favorable conformations followed by the insertion of the G93A mutation and repeat of side chain repacking. Using the SOD1^{G93A-WT} structure generated from the SOD1^{WT} structure, folding energy scores of SOD1^{WT} and SOD1^{G93A-WT} were determined using RosettaDesign.

2.2.3 Homology Modeling and Crystal Structure Analysis—The crystal structures of holo-form SOD1^{WT} and SOD1^{G93A} (PDB ID: 2C9V and 2WKO, respectively) [24, 30] were visualized using PyMol [31].

2.2.4 Transfection of HEK293T and NSC-34 Cells—Transfection of HEK293T and NSC-34 cells was performed as previously reported [14]. HEK293T cells were maintained at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium/High Glucose (DMEM/High Glucose, Thermo Scientific, Pittsburgh, PA) supplemented with 10% FBS, 100 µg/mL of streptomycin sulfate and 100 units/mL of penicillin. NSC-34 cells were maintained in DMEM supplemented with 10% FBS, 4500 mg/L glucose, 0.584 g/L L-glutamine, 3.7 g/L sodium bicarbonate, 100 µg/mL of streptomycin sulfate and 100 units/ml of penicillin. Cells were seeded on 6-well plates one day prior to transfection. Once the cells grew to 80–90% confluency, they were transfected with 3.5 µg of the appropriate plasmid via the calcium phosphate precipitation method. All samples were done in triplicate, unless explicitly stated.

2.2.5 Fluorescence Microscopy—Fluorescence microscopy was performed as previously reported [14]. Two days post-transfection, HEK293T or NSC-34 cells were visualized via fluorescence microscopy using a Vista Vision Inverted Fluorescence Microscope (VWR, Radnor, PA). Images were captured using a DC-2C digital camera. The fluorescence excitation wavelength range was between 420 and 485 nm and the emission wavelength was 515 nm. The number of cells in the fluorescence microscopy images was

manually counted. The percentage of transfected cells exhibiting SOD1-EGFP aggregates was determined by dividing the number of aggregate-containing cells by the number of total cells analyzed. Only images containing greater than 50 cells were used. Three images per well of transfected cells were used.

2.2.6 Flow Cytometric Analysis of Cellular Fluorescence—Flow cytometric analysis of cellular fluorescence was performed as previously reported [14, 32]. Two days post-transfection, the transfected HEK293T and NSC-34 cells were trypsinized, washed twice with 1× PBS and resuspended in 500µL of 1× PBS. The fluorescence intensities of the HEK293T and NSC-34 cells expressing SOD1 variant-EGFP fusion protein were measured using the C6 flow cytometer (BD Biosciences, San Jose, California). The excitation wavelength was 488 nm and the fluorescence emission was detected at 585 nm. Only GFP positive cells were used to calculate the mean cellular fluorescence. Transfection efficiency was determined by dividing the number of fluorescence positive cells by the number of total cells analyzed. Each sample was prepared in triplicate and cellular fluorescence indicates mean cellular fluorescence, unless otherwise noted. Two-sided, Student's paired t-test was used for statistical analysis of fluorescence data.

2.2.7 Total Protein Extraction and Protein Fractionation of HEK293T cells—Total protein extraction and protein fractionation of HEK293T cells were performed as previously reported [14]. 2×10^6 transfected HEK293T cells were centrifuged to obtain a cell pellet. The cell pellet was washed once with PBS buffer and lysed using 100 µL of RIPA buffer (Thermo Fisher, Pittsburgh, PA). To remove the detergent soluble fraction of proteins, the cells were incubated at 4 °C for 10 minutes. The samples were centrifuged at 4 °C and 15,500 g for 15 minutes. The supernatant was collected as a soluble fraction and kept on ice. The pellet was collected as an insoluble fraction and was then resuspended in 100 µL of RIPA buffer at 4 °C for 10 minutes. The samples were centrifuged at 4 °C and 15,500 g. The supernatant was discarded and the procedure was repeated once more. To extract the detergent-insoluble fraction of proteins, the pellet was incubated at room temperature in 100 µL of RIPA Buffer/8M Urea overnight. The sample was centrifuged at 15,500 g for 15 minutes and the supernatant was collected. For total protein lysate extraction, cell pellets were resuspended in 100 µL of RIPA Buffer/8M Urea and incubated at room temperature overnight. Protein concentrations of the soluble fraction and total protein lysate were determined using BCA kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

2.2.8 Western Blotting—Western Blotting was performed as previously reported with minor adjustments [14, 32]. Soluble fractions were diluted using RIPA and 8M Urea to 1 µg/µL after the protein concentration was determined. The final sample buffer composition of the soluble fraction was 1:3 vol/vol (RIPA:8M Urea). The insoluble fractions were diluted using the same amount of volume of buffer used to dilute the soluble fraction for that particular mutant. For total SOD1 determination, the final concentration for the lysate was adjusted to account for varying transfection efficiencies among the variants. 3 µL of total protein, soluble or insoluble protein lysate were boiled in sample loading buffer containing DTT and electrophoresed on 12% SDS-PAGE Gel. Samples were transferred onto a nitrocellulose membrane for one hour and washed with 1× TBS-T three times for 5 minutes.

The membrane probed with the primary anti-SOD1 antibody diluted in TBS-T for 1 hour at RT (1:5000). Samples were washed three times for 5 minutes in TBS-T and probed with the secondary antibody conjugated with HRP for 1 hour (1:10000). Bands were detected using ECL Prime (GE Healthcare, Sweden).

3. RESULTS AND DISCUSSIONS

3.1 Determination of key factors responsible for the destabilization of SOD1^{G93A} using *in silico* techniques

It is well known that SOD1^{G93A} forms intracellular aggregates in mammalian cells. This is likely due to the reduced conformational stability caused by glycine to alanine mutation at position 93 (G93A) [24, 26]. As the first step to reduce SOD1^{G93A} aggregation, we investigated key factors responsible for SOD1^{G93A} destabilization. In order to evaluate the effects of G93A mutation on conformational stability, we constructed a structural model of SOD1^{G93A} (SOD1^{G93A-WT}) by introducing G93A mutation into the SOD1^{WT} structure.

Using RosettaDesign, we calculated the conformational stability (folding energy score;

G_f) of SOD1^{WT} and SOD1^{G93A-WT}. The folding energy score of SOD1^{G93A-WT} (-122 kcat/mol) is substantially higher than that of SOD1^{WT} (-541 kcal/mol), indicating that SOD1^{G93A-WT} is conformationally less stable than SOD1^{WT} (Table 1). Then, we investigated which score plays a key role in increasing the folding energy score among twenty individual scores, summed for the final folding energy score. It is noteworthy that the G93A mutation substantially increases fa_{rep} score (from 114 to 551 kcal/mol) comparable to the increase in the final folding energy score (Table 1). Since fa_{rep} score is a measure of the repulsive portion of the Lennard-Jones Potential, such an increase indicates certain atoms are closer than their optimal distance leading to the SOD1^{G93A} destabilization. Complementary to the computational stability analysis, we also performed visual inspection of the crystal structures of SOD1^{WT} (PDB ID:2C9V) and SOD1^{G93A} (PDB ID:2KWO). Although G93 in SOD1^{WT} does not have any close contact with a neighboring protein backbone, a methyl group in the side chain of A93 in SOD1^{G93A} comes in close contact with the neighboring protein backbone (Figure S1A and B). Therefore, the combination of computational stability analysis and visual inspection of protein crystal structure suggest that the repulsive interactions between the side chain of A93 and the protein backbone is responsible for the SOD1^{G93A} destabilization.

3.2 Mutation of residues significantly displaced upon the G93A mutation followed by cell-based screening of SOD1^{G93A} variants

To our knowledge, no rational design strategy has been reported that redesigns a protein backbone in order to eliminate repulsive interactions between a side chain and a protein backbone. Therefore, we sought an alternative way to reduce SOD1^{G93A} aggregation by mutating the residues significantly displaced upon the G93A mutation. When the protein backbones of SOD1^{WT} and SOD1^{G93A} are overlaid in PyMOL, there are displacements at multiple sites, including the SOD1^{G93A} backbone shift from the SOD1^{WT} backbone (Figure S1 A), as well as side chain displacements (Figure 1A to F) likely due to the repulsive interactions between the side chain of A93 and a protein backbone (Figure S1 B). Therefore, we hypothesize that these side chain displacements play an important role in SOD1^{G93A}

aggregation, and so mutation of the amino acids significantly displaced upon the G93A mutation will reduce protein aggregation. We focused on twelve residues within a 5Å distance from the mutated A93 (I35, K36, G37, L38, T39, E40, A89, D90, K91, D92, V94, and A95). To our knowledge, PyMOL software does not provide quantitative measure of side chain displacements. Therefore, we performed visual inspection of the twelve residues in the crystal structures of both SOD1^{WT} and SOD1^{G93A} (Figure S1C and D). We found three residues that fall under these specific criteria, K36, E40 and K91 (Figure 1A to F). The relative level of side chain displacement of the three residues is K91 ~ K36 > E40, though the differences are small. It is noteworthy that no side chain of these residues directly contacts the side chain of A93. In order to avoid the perturbation of local polarity, we generated seven SOD1^{G93A} variants containing a mutation of each of three charged amino acids (K36, E40, and K91) into different charged amino acids (K36E; E40R, E40K, and E40D; K91E, K91D, and K91R). One additional mutation from a charged amino acid into a bulky, hydrophobic one (K36L) was also a non-conservative mutation.

In order to identify SOD1^{G93A} variants with a reduced aggregation propensity in mammalian cells, we used the mammalian cell-based aggregation assay correlating misfolding/aggregation propensity to the extent of reduction in cellular fluorescence [14, 32]. Briefly, an EGFP was fused to C-terminus of SOD1^{WT}, SOD1^{G93A}, or each of SOD1^{G93A} variants containing the aforementioned additional mutation. Both cell lines (HEK293T and NSC-34) were used to express an EGFP fusion of SOD1^{variant}. HEK293T is a cell line used extensively in cell biology experiments and biologics production, and NSC-34 is a mouse neuroblastoma commonly used for fALS research [33–38]. Both HEK293T and NSC-34 cells were transfected and harvested two days post-transfection for flow cytometric analysis. Only three SOD1^{G93A} variants (SOD1^{G93A/K36E}, SOD1^{G93A/K91E} and SOD1^{G93A/K91D}) exhibited a significant increase in the cellular fluorescence in both cell lines ($P < 0.01$ or $P < 0.05$) (Figure 2). As demonstrated previously, such an increase in the cellular fluorescence is very likely attributed to a significant reduction in the aggregation propensity [14, 22, 32].

Although absolute cellular fluorescence is a key indicator of whether the target protein has exhibited a significant increase in aggregation propensity, total protein expression must also be taken into account to dismiss any notion that the cellular fluorescence increase resulted from increased protein expression due to other factors, such as reduced degradation. To determine relative total SOD1 protein expression for each SOD1^{variant}, the total protein lysate was extracted from HEK293T cells and analyzed using western blotting. The mean cellular fluorescence values for HEK293T were adjusted by determining the ratio of the band intensities of each variant to SOD1^{WT} to determine the effect of total SOD1 expression (Figure S2). Even when accounting for variation in total protein expression, all three variants (SOD1^{G93A/K36E}, SOD1^{G93A/K91E} and SOD1^{G93A/K91D}) exhibited a substantial increase in mean fluorescence. In contrast, SOD1^{G93A/K36L} did not exhibit any increase in the cellular fluorescence in both cell lines (Figure 2A and B) suggesting that the mutation of a charged amino acid (K36) into a bulky, hydrophobic amino acid is not effective in reducing the aggregation propensity of SOD1^{G93A}. Therefore, we conclude that the protein expression level change due to the reduced protein degradation is not a dominant factor for the reduced intracellular aggregation of the three promising SOD1^{G93A} variants

(SOD1^{G93A/K36E}, SOD1^{G93A/K91E} and SOD1^{G93A/K91D}). The three promising SOD1^{G93A} variants and several other variants were further subjected to fluorescence microscopic analysis.

3.3 Fluorescence microscopic analysis of the promising SOD1^{G93A} variants expressed in HEK293T cells

Fluorescence microscopy of HEK293T cells expressing an EGFP fusion of SOD1^{variant} at two days post-transfection was performed to confirm whether the trends observed from the fluorescence intensity measurements were due to changes in intracellular aggregation of the SOD1 variants. Similar approaches have been performed in order to visualize intracellular aggregation of mutant SOD1 [14, 39–42]. From visualization of the EGFP fusion of SOD1^{variant} within HEK293T cells, it is apparent that SOD1^{G93A} exhibits significant intracellular aggregation SOD1^{G93A} (Figure 3 *G93A panel*), while SOD1^{WT} does not exhibit any intracellular aggregation (Figure 3 *WT panel*). As expected, the HEK293T cells expressing each of three promising SOD1^{G93A} variants (SOD1^{G93A/K36E}, SOD1^{G93A/K91D}, and SOD1^{G93A/K91E}) exhibited substantially reduced levels of intracellular aggregation in comparison to SOD1^{G93A} (Figure 3 *G93A/K36E*, *G93A/K91E*, and *G93A/K91D panels*), which is consistent with the results found in flow cytometric analysis (Figure 2A and B). The other SOD1^{G93A} variants that do not exhibit a significant increase in the cellular fluorescence in both cell lines, including SOD1^{G93A/K36L}, show significant levels of intracellular aggregation (Figure S3). For SOD1^{G93A} and the three promising SOD1^{G93A} variants (SOD1^{G93A/K36E}, SOD1^{G93A/K91D}, and SOD1^{G93A/K91E}), the fraction of cells exhibiting intracellular aggregation was determined (Figure 4). For the three promising SOD1^{G93A} variants, there was at least a 2.5 fold decrease in the fraction of cells exhibiting intracellular SOD1 aggregation ($p < 0.01$ for all samples compared to SOD1^{G93A}) (Figure 4). In particular, the introduction of K36E into SOD1^{G93A} almost completely eliminates intracellular aggregation in HEK293T cells.

3.4 Determination of the aggregation propensity of three SOD1^{G93A} variants by using cell lysate fractionation

Using a simple protein engineering strategy, mammalian-cell based screening, and fluorescence microscopic analysis, three SOD1^{G93A} variants (SOD1^{G93A/K36E}, SOD1^{G93A/K91D}, and SOD1^{G93A/K91E}) with a reduced aggregation inside mammalian cells were identified. In order to more quantitatively evaluate relative aggregation propensity of SOD1 variants, we performed cell fractionation analysis of HEK293T cells expressing the EGFP fusion of SOD1^{WT}, SOD1^{G93A}, SOD1^{G93A/K36E}, SOD1^{G93A/K91D}, and SOD1^{G93A/K91E} (Figure 5). As described previously [14], detergent soluble and detergent-insoluble proteins for the aforementioned SOD1 constructs were extracted from HEK293T cells two days post transfection and their western-blot band intensities were compared (Figure S4). Relative aggregation propensity is the ratio of insoluble fraction band intensity and the soluble fraction band intensity, as described previously [14, 26, 40, 43]. To normalize bands from different membranes, the SOD1^{G93A} aggregate propensity was set to 1.25 and the insoluble band intensities for all other samples were adjusted. All three novel SOD1^{G93A} variants showed a significant reduction ($p < 0.05$) in the relative aggregation propensity compared to that of SOD1^{G93A}. This is consistent with the trend observed in the

fluorescence microscopic and flow cytometric analyses described earlier. From the visual inspection of the aligned structures of SOD1^{WT} and SOD1^{G93A}, we found that the relative level of side chain displacement of the three residues is K91 ~ K36 > E40 (Figure 1). Considering that mutations at K91 and K36 are more effective in reducing the aggregation propensity than mutations at E40, we speculate that the level of side chain displacement may be a factor identifying promising residues to mutate. We are currently working on establishment of a more quantitative technique to measure the level of side chain displacement.

3.5 Aggregate formation rate of the novel SOD1^{G93A} variants in mammalian cells

So far, all three variants (SOD1^{G93A/K36E}, SOD1^{G93A/K91E} and SOD1^{G93A/K91D}) showed a reduced propensity to aggregate within mammalian cells compared to SOD1^{G93A}. Next, we compared the rate of aggregation formation within HEK293T cells of the three novel SOD1^{G93A} variants. The fraction of cells exhibiting intracellular aggregates of SOD1 variant was determined every 24 hours for three days using fluorescence microscopy (Figure 6). As expected, the three variants (SOD1^{G93A/K36E}, SOD1^{G93A/K91E} and SOD1^{G93A/K91D}) form intracellular aggregates significantly slower than SOD1^{G93A}. Combined with results indicating that the change in protein expression level is not a dominant factor for the enhanced cellular fluorescence, these findings suggest that there is less inclination for the three variants to aggregate over the given period of time.

In order to investigate whether the three SOD1^{G93A} variants are conformationally more stable than original SOD1^{G93A}, the RosettaDesign was used. Compared to a significant change in both scores upon G93A mutation in SOD1^{WT} (Table 1), the change in both scores made by each of the mutations (K36E, K91E, and K91D) is negligible (Table 1), which may be explained by the fact that neither the side chain of K36 nor K91 directly interact with the side chain of A93 causing the repulsive interaction with the neighboring protein backbone. These calculation results suggest that the reduced aggregation propensities of the three variants (SOD1^{G93A/K36E}, SOD1^{G93A/K91E} and SOD1^{G93A/K91D}) do not result from enhanced conformational stability.

Instead, we speculate that the alteration of surface charge of SOD1^{G93A} is a reason for the reduced aggregation propensity. It was reported that the supercharging strategy, introduction of multiple charged residues on the surface of a protein, was successfully applied to reduce protein aggregation without affecting conformational stability [44, 45]. All three mutations (K36E, K91E, and K91D) lead to reduction of the net charge by 2. There is a possibility that the negative charge at residue 36 or 91 enhances inter-molecular repulsive interactions resulting in the reduced aggregation propensity.

4. CONCLUSIONS

Understanding how to systematically reduce intracellular protein aggregation has substantial implications for biopharmaceutical production and disease pathology. To mitigate complications that arise from protein aggregate development within mammalian cells, we employed simple protein design and cell-based aggregation assay to identify mutations reducing intracellular aggregate formation. The aggregation-prone mutant SOD1^{G93A} was

used as a model system. The G93A mutation causes the repulsive interaction with the neighboring protein backbone leading to backbone shift and multiple side chain displacements. A panel of SOD1^{G93A} variants containing a mutation at three residues (K36, E40, and K91) of which side chain were significantly displaced upon the G93A mutation were generated and subjected to cell-based screening. By examining the fraction of cells exhibiting intracellular aggregation and comparing the aggregation propensity of SOD1^{G93A} variants, three novel SOD1^{G93A} variants (SOD1^{G93A/K36E}, SOD1^{G93A/K91D} and SOD1^{G93A/K91E}) with a reduced aggregation propensity inside mammalian cells were identified. Furthermore, the aggregation rate of these variants is substantially lower than that of SOD1^{G93A}. Our findings demonstrate that a simple protein engineering strategy of mutating residues significantly displaced upon the original mutation in combination with cell-based screening is a practical way to identify second-site mutations reducing aggregation-propensity in mammalian cells.

Although the exact mechanism underlying reduced aggregation propensity of the novel variants should be further studied, the simplicity of techniques and schemes employed in this study make it a practical option to engineer other proteins in order to reduce the aggregate propensity in mammalian cells. In particular, the application to therapeutic protein engineering would be possible. When therapeutic proteins (cytokines and antibodies) are expressed inside mammalian cells, the reduced aggregation propensity and/or enhanced solubility can improve product yield. Even for therapeutic proteins that are secreted into media, intracellular aggregation will reduce the amount of proteins secreted. In the early stages of new therapeutic protein development, mutations and/or fusions are usually applied to improve their therapeutic activities and binding affinity. In the course of such engineering, it is possible to identify antibody mutants with improved therapeutic properties, but enhanced aggregation propensity due to the mutation(s) introduced. In such cases, the techniques and schemes described here may be useful to reduce the aggregation propensity without affecting other critical therapeutic activities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors appreciate Dr. Haining Zhu (University of Kentucky) for providing the pEGFP-N3-SOD1^{WT} plasmids. This work was supported by a National Institutes of Health Grant (R21NS069946) and a Korea CCS R&D Center (KCRC) grant (2013M1A8A1038187). Additional support was provided by the TRANE Fellowship (S.G.).

Abbreviations

ALS	Amyotrophic lateral sclerosis
DMEM	Dulbecco's modified Eagle's medium
EGFP	enhanced green fluorescent protein
FBS	fetal bovine serum

GFP	green fluorescent protein
HEK	human embryonic kidney cell
SOD1	human copper/zinc superoxide dismutase

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Highlights

- Protein aggregation mediated by mutation-induced structural changes is proposed.
- Cell-based assays are effective in selecting mutants with reduced aggregation.
- Mutation of perturbed residues reduces protein aggregation in mammalian cells.
- Strategies described here would be a practical option to reduce protein aggregation.

Figure 1. Residues of SOD1^{G93A} substantially displaced compared to SOD1^{WT}. Residues K36 (purple) and G93 (red) (A); E40 (orange) and G93 (red) (B); and K91 (yellow) and G93 (red) (C) within crystal structure of the holo-form of SOD1^{WT} (PDB ID: 2C9V). Residues K36 (purple) and A93 (blue) (D); E40 (orange) and A93 (blue) (E); and K91 (yellow) and A93 (blue) (F) within crystal structure of the holo-form of SOD1^{G93A} (PDB ID: 2WKO).

Figure 2.

The mean cellular fluorescence of the transfected HEK293T (A) and NSC-34 (B) cells expressing EGFP fusion of SOD1 variants. (C) The SOD1 bands of the transfected HEK293T cells expressing the SOD1 variants. Values and error bars represent mean cellular fluorescence and standard deviations, respectively (n=3). In order to determine whether the mean cellular fluorescence of the SOD1 double mutants is greater than that of SOD1^{G93A} in each cell line, two-sided Students t-tests were applied to the data (* $p<0.05$; ** $p<0.01$).

Figure 3.

The fluorescence microscopic images of the transfected HEK293T cells expressing EGFP fusion of SOD1 variants. The images of transfected HEK293T cells expressing EGFP fusion of SOD1^{WT}, SOD1^{G93A}, and SOD1^{G93A} double mutants were taken at 2 days post-transfection. (white arrows: intracellular SOD1 mutant aggregates; scale bar: 50 μm)

Figure 4.

The fraction of the transfected HEK293T cells exhibiting SOD1 aggregates. The number of cells exhibiting intracellular SOD1 aggregates was determined by analyzing fluorescence microscopy images of the transfected cells expressing EGFP fusion of four SOD1 variants ($\text{SOD1}^{\text{G93A}}$, $\text{SOD1}^{\text{G93A/K36E}}$, $\text{SOD1}^{\text{G93A/K91D}}$ and $\text{SOD1}^{\text{G93A/K91E}}$) and SOD1^{WT} at 2 days post-transfection ($p < 0.01$ for all samples compared to $\text{SOD1}^{\text{G93A}}$).

Figure 5.

The relative aggregation propensity of SOD1 proteins at 48 h. In order to determine whether the SOD1 variant aggregation propensity is smaller than that of SOD1^{G93A}, two-sided Students t-tests were applied to the data (*, $p < 0.05$) (n=3).

Figure 6.
The fraction of the transfected HEK293T cells exhibiting intracellular SOD1 aggregates determined at 0, 24, 48 and 72 hours post-transfection.

Table 1Scoring summary of SOD1^{WT} and SOD1^{G93A_WT} variants

SOD1 variant	G_f (kcal/mol)	fa_rep (kcal/mol)
SOD1 ^{WT}	-541	114
SOD1 ^{G93A_WT}	-122	551
SOD1 ^{G93A/K36E_WT}	-132	511
SOD1 ^{G93A/K91D_WT}	-127	510
SOD1 ^{G93A/K91E_WT}	-128	511