

Oxidized glutathione stimulated the amyloid formation of α -synuclein

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Abstract α -Synuclein is the major filamentous constituent of Lewy bodies found in Parkinson's disease (PD). The amyloid formation of α -synuclein was significantly facilitated by oxidized glutathione (GSSG) as the lag period of the aggregation kinetics was shortened by 2.5-fold from its absence. Reduced glutathione (GSH), on the other hand, did not influence the lag phase although it increased the final amyloid formation. The GSSG stimulation was specific for not only α -synuclein but also its intactness. The preferred GSSG interaction of α -synuclein to GSH was also demonstrated with dissociation constants of 0.53 and 43.5 mM, respectively. It is suggested that the oxidative stress favoring the GSSG generation from GSH could result in the augmented amyloid formation of α -synuclein, which ought to be related to the pathogenesis of PD.
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Key words: α -Synuclein; Glutathione; Amyloid formation; Protein aggregation; Oxidative stress; Parkinson's disease

1. Introduction

Oxidative stress has been implicated in various age-related neurodegenerative disorders [1,2]. Parkinson's disease (PD) shows clinical manifestations of resting tremor, slowness of initial movement, rigidity, and general postural instability. The disease has been pathologically characterized by progressive loss of the dopaminergic neurons in the substantia nigra and the presence of eosinophilic intraneuronal inclusions called Lewy bodies [3]. α -Synuclein has been considered to be a pathological component of PD because not only it constitutes the Lewy bodies as the major filamentous component but also two independent missense mutations in its gene have been identified from a few pedigrees of familial PD [4–6]. The resulting proteins contain either proline or threonine replacing alanine residues at positions 30 and 53 (Ala30Pro and Ala53Thr), respectively [5,6]. When the protein was overexpressed in mice and flies, the animals exhibited several characteristics reminiscent of human PD including protein deposition in the brain, dopaminergic neuronal loss, and deficits in motor activity [7,8]. Although physiological function remains elusive, the protein has been intensively examined with respect to the amyloid formation and its implication in the pathogenesis of PD [9,10].

There have been several reports indicating that oxidative insults have influenced α -synuclein to be more aggregative.

Metals such as copper and iron gave rise to sodium dodecyl sulfate (SDS)-resistant oligomers and/or aggregates of the protein in the presence of hydrogen peroxide [11,12]. Nitration of α -synuclein also caused protein self-oligomerization via *o,o'*-dityrosine crosslink formation [13]. Oxidative stress has emerged from imbalance between generation of reactive oxygen species (ROS) and antioxidative defense mechanism [1,14]. Brain is especially susceptible to ROS because not only the tissue consumes a large proportion of oxygen taken into the body but also it contains higher amounts of membrane lipids with polyunsaturated fatty acyl chains and transition metals including iron (and copper) which could be involved in generation of hydroxyl radical in the presence of hydrogen peroxide [1,14]. In addition, the nervous system is poor at eliminating ROS because of its reduced catalase activity with moderate amounts of superoxide dismutase and glutathione peroxidase [14–16]. In PD, the oxidative stress might be more serious because hydrogen peroxide level is suggested to be increased with the augmented breakdown of dopamine by monoamine oxidase [16,17], whereas the amount of reduced glutathione (GSH), one of a few low molecular weight antioxidants synthesized by the cell, has been substantially decreased by 40% from 0.75 to 0.45 $\mu\text{mol/g}$ wet weight of the affected substantia nigra [18]. Intriguingly, however, oxidized form of glutathione (GSSG) was insignificantly increased from 0.0024 to 0.0031 $\mu\text{mol/g}$ wet weight [18]. In this report, we have examined direct molecular interaction between α -synuclein and GSH/GSSG in order to assess the relationship between oxidative stress and abnormal protein aggregation leading to the eventual amyloid formation.

2. Materials and methods

2.1. Materials

Reduced and oxidized glutathiones were purchased from Sigma. Thioflavin-T, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), GSH-Sepharose, epoxy-activated Sepharose, thrombin, and endoproteinase Asp-N were also obtained from Sigma. Carbon-coated copper grid and centrifugal filter (Ultrafilter[®]-MC) were provided by Ted Pella Inc. and Millipore, respectively. Uranyl acetate was from Electron Microscope Science.

2.2. Preparation of the synucleins

Overexpressed recombinant α - and β -synucleins in *Escherichia coli* were purified according to the procedures described previously [19,20]. The C-terminally truncated α -syn(1–97) was prepared with endoproteinase Asp-N treatment as described elsewhere [21]. The N-terminally truncated α -syn(61–140) was overexpressed as a glutathione-S-transferase fusion protein. The recombinant protein was purified with glutathione-Sepharose chromatography using 10 mM glutathione in 50 mM Tris-Cl, pH 7.5, as the elution buffer. Following thrombin digestion (10 units/mg protein) for 4 h at 24°C, α -syn(61–140) was subjected to Q-Sepharose anion-exchange chromatography under a linear gradient between 0 and 1 M NaCl in 20 mM Tris-Cl, pH

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7.5. The purified protein was dialyzed against 20 mM MES, pH 6.5, and kept at 1.2 mg/ml in -30°C .

2.3. Analysis of protein aggregation

Protein aggregations of α - and β -synucleins in the presence and absence of 2 mM each of GSH and GSSG were examined with turbidity by observing absorbance at 405 nm. Following ultracentrifugation at $100\,000\times g$ for 10 min to remove any protein clumps which could act as a nucleation center during the aggregation, either α -synuclein (100 μM) or β -synuclein (131 μM) was mixed with the glutathiones in 20 mM MES, pH 6.5, to a final volume of 1 ml and incubated at room temperature with continuous shaking. Amyloid formation of the protein aggregates was evaluated with thioflavin-T binding fluorescence [22]. During the incubation, small aliquots (5 μl) were combined with 5 μM thioflavin-T in 50 mM glycine, pH 8.5, to a final volume of 300 μl and the fluorescences were measured at 482 nm with an excitation at 446 nm (LS55 Luminescence Spectrometer, Perkin-Elmer). Morphological appearances of the amyloids were also examined with a transmission electron microscope (H7100, Hitachi). Aliquots (5 μl) of the aggregates were adsorbed onto carbon-coated copper grid (300 mesh) and air-dried for 1 min. After negative staining with 2% uranyl acetate for another 1 min, the aggregates were observed with the electron microscope.

For another set of experiments to localize the glutathione interaction site(s), the synucleins such as α -synuclein, β -synuclein, α -syn(1–97), and α -syn(61–140) were incubated at a final concentration of 90 μM with 2 mM GSH/GSSG in 20 mM MES, pH 6.5, for 96 h at room temperature with continuous upside-down mixing (Rotamix SLRM1, Seoulin Bioscience). Aliquots (20 μl) of the 150 μl final mixtures were mixed with 5 μM thioflavin-T in 50 mM glycine, pH 8.5. Fluorescences of the mixtures (100 μl) were measured at 485 nm with excitation at 440 nm (FL500 Microplate Fluorescence Reader, Bio-Tek Instruments).

2.4. Determination of K_d between α -synuclein and GSH/GSSG

Dissociation constants between α -synuclein and GSH/GSSG were determined by employing immobilized α -synuclein on Sepharose gel [20]. Derivatization of α -synuclein to the gel was carried out with pre-swollen epoxy-activated Sepharose 6B in 0.1 M potassium phosphate, pH 7.25. After packing the gel into a column, α -synuclein (4.3 mg/6 ml) was subjected to trace enrichment in the phosphate buffer by using a peristaltic pump at a flow rate of 0.25 ml/min for 85 h at room temperature. The amount of immobilized protein was estimated as 1 mg/ml of the gel. After blocking with 1 M ethanolamine, the α -synuclein-Sepharose was kept in the phosphate buffer at 4°C .

The α -synuclein-Sepharose corresponding to the final protein concentration of 5 μM was incubated with various concentrations of GSH/GSSG in 20 mM MES, pH 6.5, for 30 min at 30°C in a Thermomixer compact (Eppendorf) at 1000 rpm. Unbound GSH/GSSG was separated from the bound ones with a centrifugal filter with pore size of 0.22 μm (Ultrafilter[®]-MC, Millipore) at $250\times g$ for 1 min. After washing the gel with 200 μl of the buffer, the bound glutathiones were eluted with 1 M NaCl. Following 10 min of incubation, the glutathiones were recovered with the centrifugation at $250\times g$ for 2 min. The amount of the bound GSH was quantitated with 0.4 mM DTNB by measuring absorbance of its reduced product, 5-thio-2-nitrobenzoate (TNB), at 405 nm using a microplate reader (Elx 808, Bio-Tek Instruments) [23]. The recovered GSSG was enzymatically quantified with GSSG reductase in the presence of 0.4 mM DTNB and 0.17 mM NADPH according to the procedure described elsewhere [17,18]. Double reciprocal plots between total and bound GSH/GSSG were drawn to obtain the dissociation constants.

3. Results and discussion

Amyloid formation of α -synuclein in the presence and absence of GSSG/GSH was evaluated with the thioflavin-T binding fluorescence. The amyloid formation of α -synuclein was significantly facilitated in the presence of 2 mM GSSG as the lag period of the protein aggregation was shortened by 2.5-fold from its absence (Fig. 1A). While α -synuclein itself started to form the aggregates after 125 h of prolonged lag period, GSSG reduced the lag to around 50 h. On the other

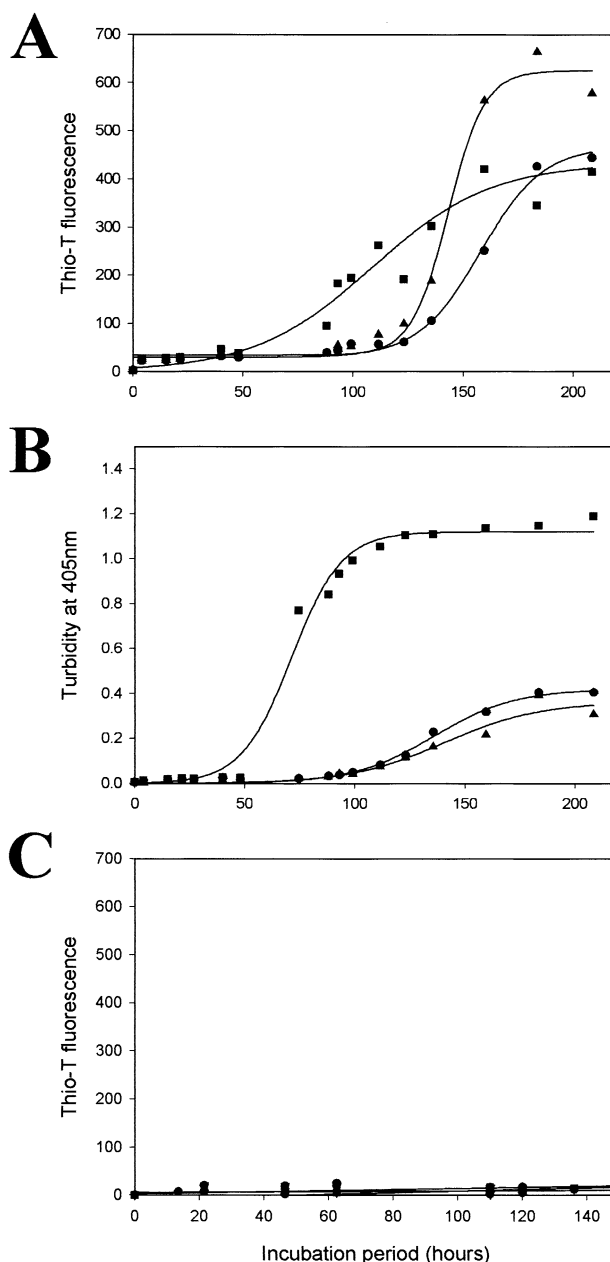


Fig. 1. Protein aggregations of α - and β -synucleins in the presence and absence of GSH/GSSG. α -Synuclein (100 μM) was incubated with 2 mM each of GSH (\blacktriangle) and GSSG (\blacksquare) and the aggregations were analyzed with the thioflavin-T binding fluorescence (A) and the turbidity (B). Aggregation of α -synuclein by itself (\bullet) is shown in each panel as a control. The aggregations of β -synuclein (131 μM) were also examined with the thioflavin-T binding fluorescence (C) in the absence (\bullet) and presence of GSH (\blacktriangle)/GSSG (\blacksquare).

hand, GSH did not affect the lag although it increased the final fluorescence by 40% compared to the aggregation by α -synuclein alone. The GSH effect, however, was not observed unless the amyloid of α -synuclein was started to form by itself. GSSG/GSH alone did not give rise to the fluorescence even after prolonged incubation (data not shown). These data indicate that the GSSG interaction to α -synuclein could provide a nucleation center for the amyloidosis of the protein while the GSH interaction appeared to depend on the prior aggregation of α -synuclein. Several independent obser-

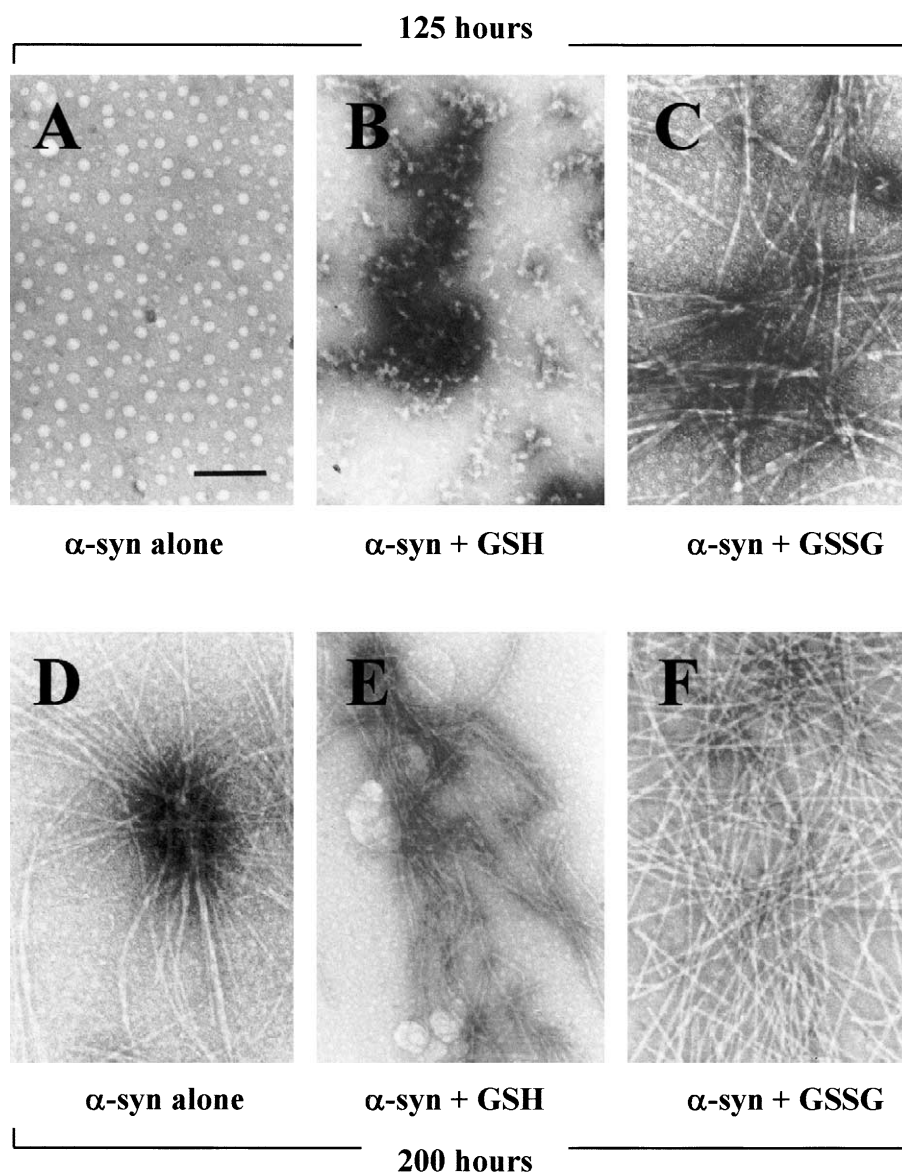


Fig. 2. Morphologies of the protein aggregates. The aggregates of α -synuclein prepared in the absence (A, D) and presence of 2 mM GSH (B, E) or GSSG (C, F) were visualized with electron microscope. The protein aggregates were collected at two different time points of 125 h (A, B, C) and 200 h (D, E, F) following the incubation. Scale bar represents 0.5 μ m.

vations consistently demonstrated that the GSH stimulation has never proceeded to the amyloid formation of α -synuclein by itself. When the aggregations were simultaneously evaluated with turbidities, GSSG exhibited the most dramatic stimulation with prominent shortening of the lag period while the aggregations obtained with and without GSH were slowly increased to lesser extents (Fig. 1B). In the case of β -synuclein, the thioflavin-T binding fluorescence was not observed at all even in the presence and absence of GSSG/GSH, indicating that the stimulatory GSSG effect on the amyloid formation was selective to α -synuclein (Fig. 1C).

Formation of fibrous structures during the GSSG-induced α -synuclein aggregation was morphologically analyzed with a transmission electron microscope. After 125 h of incubation, the aggregation obtained with GSSG clearly showed the fibrous structures (Fig. 2C) while the aggregates generated in the presence and absence of GSH did not form the amyloids yet (Fig. 2A and B). The aggregates prepared with GSH

showed rather worm-like structures (Fig. 2B). All the final aggregates, however, after the prolonged incubation of 200 h with and without GSSG/GSH gave rise to the common fibrous structures (Fig. 2D–F). It was, therefore, clear that the GSSG stimulation of α -synuclein led to the authentic amyloid formation.

The enhancing GSSG effect on the amyloid formation of α -synuclein was further examined with both N-terminally and C-terminally truncated α -synucleins such as α -syn(61–140) and α -syn(1–97), respectively, to localize the GSSG interaction site(s). Intriguingly, it turned out that intactness of α -synuclein was essential for the stimulatory effect of GSSG. The synucleins (90 μ M) including α -synuclein, β -synuclein, α -syn(1–97), and α -syn(61–140) were incubated with 2 mM each of GSH/GSSG in 20 mM MES, pH 6.5, at room temperature. Following 96 h of incubation, the amyloid formation was estimated with the thioflavin-T binding fluorescence (Fig. 3). In this new set of experiments, the protein aggregation of

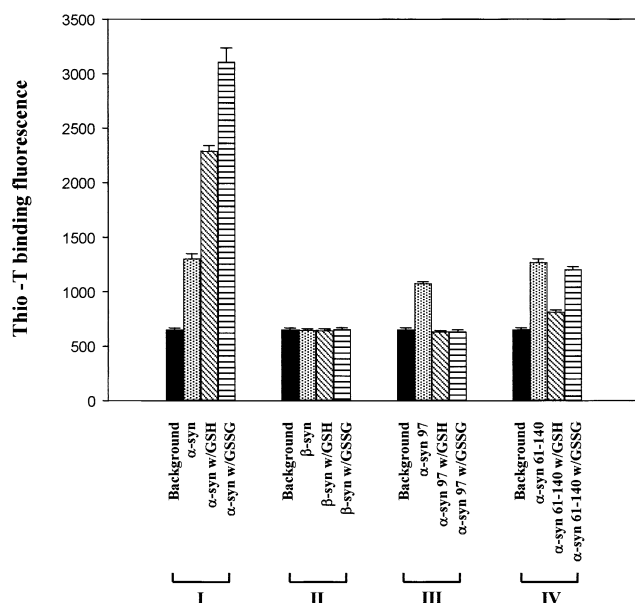


Fig. 3. Effects of GSH/GSSG on the amyloid formation of various synucleins. The synucleins such as α -synuclein (I), β -synuclein (II), α -syn(1–97) (III), and α -syn(61–140) (IV) were incubated with or without 2 mM GSH/GSSG for 96 h at room temperature in 20 mM MES, pH 6.5, at a final concentration of 90 μ M. The amounts of amyloids were estimated with the thioflavin-T binding fluorescence. Each set of experiments consists of four columns representing the fluorescences of background and protein aggregations obtained in the absence and presence of GSH/GSSG as indicated on the bottom of the panel.

intact α -synuclein was stimulated by 3.8- and 2.5-fold in the presence of GSSG and GSH, respectively. At the same time point, however, β -synuclein again did not form the amyloid even in the presence and absence of GSSG/GSH. For the C-terminally truncated α -synuclein, both GSH and GSSG did not stimulate the amyloid formation but rather inhibited the protein aggregation of α -syn(1–97). In the case of N-terminally truncated α -syn(61–140), GSSG did not influence the aggregation process while GSH suppressed the aggregation by 75%. Although GSH has been shown to interact with all three proteins of α -synuclein, α -syn(1–97), and α -syn(61–140), the enhancing effect on the fluorescence was observed only with the intact protein whereas the aggregations of the other truncated proteins were suppressed. For the GSSG interaction, the N-terminal region of α -synuclein was necessary because the aggregation of α -syn(61–140) was not affected by the oxidized glutathione. To exhibit the stimulatory effect, however, the acidic C-terminus seemed to be also required because the aggregation of α -syn(1–97) was inhibited instead while that of the intact molecule was dramatically stimulated by GSSG. Requirement of the intactness for the stimulation could be illustrated by either multiple ligand binding to α -synuclein or alterations in residual structures of the protein, if any. Based on these data, we could reach to a conclusion that the stimulated amyloid formation by the oxidized glutathione has been selective to not only α -synuclein but also its intactness.

To understand physiological and/or pathological implications of the GSSG/GSH interaction of α -synuclein, actual dissociation constants between the molecules were examined by utilizing the immobilized α -synuclein. Dissociation con-

stant between α -synuclein and GSSG was estimated as 0.53 mM, which has exhibited much higher affinity than GSH because the reduced glutathione gave rise to a K_d of 43.5 mM (Fig. 4). This apparent 82-fold difference in the dissociation constants would make the GSSG interaction of α -synuclein considered to be pathologically relevant while the GSH interaction might not be expected even under a physiological condition. The low affinity of GSH was also reflected in the aggregation kinetics of α -synuclein, where the GSH enhancement on the amyloid formation was dependent upon the preceding aggregation of α -synuclein. Since the aggregation of α -synuclein might not occur under normal condition in vivo, the enhancing GSH effect could be neglected from a physiological point of view. The reduced glutathione, however, could participate in the amyloid formation under a condition in which the α -synuclein aggregation becomes favorable. In the case of GSSG, its binding to α -synuclein with the higher affinity and resulting structural alterations would make the protein more aggregative as observed in the aggregation kinetics of the GSSG-stimulated amyloid formation (Fig. 1A and B). Alternatively, GSSG could be also structurally modified by the protein, which might in turn influence the aggregation process as well. Since it was reported that GSSG has transformed into a state of gel in the presence of organic solvent [24], possible formation of any suprastructure among GSSG molecules could facilitate α -synuclein to participate in the aggregation process. Hence, mutual influences between α -synuclein and GSSG could be responsible for the stimulated amyloid formation.

Currently, the decreased level of GSH in PD has been investigated in terms of imposing oxidative stress to the degenerating dopaminergic neurons. In this study, we have examined direct molecular interaction between α -synuclein and GSSG as a possible consequence of the reduced GSH level and the augmented hydrogen peroxide generation via the enhanced dopamine breakdown in the disease. In fact, dopamine breakdown has been shown to increase the GSSG level as the GSH was decreased in rats [17]. It has been puzzling, how-

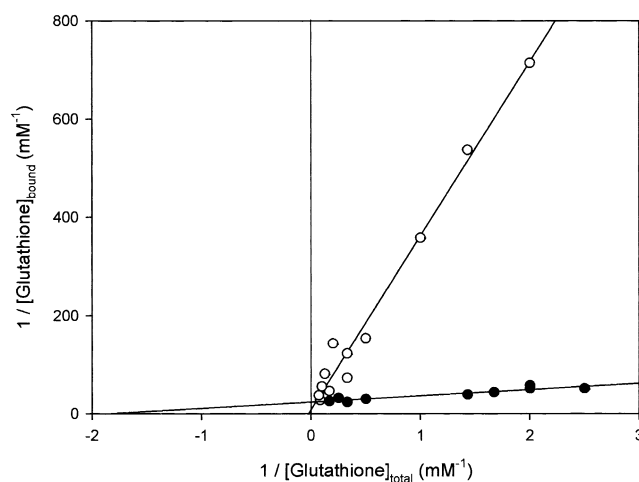


Fig. 4. Double reciprocal plots to obtain K_d between α -synuclein and GSH/GSSG. The immobilized α -synuclein (5 μ M) was incubated with various concentrations of GSH (○) and GSSG (●). The bound GSH/GSSG were separated from their free forms by using a centrifugal filter. Following quantitation, amounts of the bound and total glutathiones (GSH/GSSG) were subjected to the double reciprocal plots.

ever, that the GSSG level in the substantia nigra of PD brain was insignificantly increased while the GSH level was dramatically decreased [18]. Nevertheless, our results could indicate that the insignificant increase of GSSG may have a pathological meaning more than expected because GSSG could affect α -synuclein more effectively than GSH in vivo for the eventual production of amyloids. In any case, a conclusion obtained from this study is that the oxidative stress favoring the GSSG formation from GSH especially in PD could result in the augmented amyloid formation of α -synuclein, which ought to be related to pathogenesis of the disease.

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