The Active Site Cysteine of the Proapoptotic Protein Glyceraldehyde-3-phosphate Dehydrogenase Is Essential in Oxidative Stress-induced Aggregation and Cell Death

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Recent studies have revealed that the redox-sensitive glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is involved in neuronal cell death that is triggered by oxidative stress. GAPDH is locally deposited in disulfide-bonded aggregates at lesion sites in certain neurodegenerative diseases. In this study, we investigated the molecular mechanism that underlies oxidative stress-induced aggregation of GAPDH and the relationship between structural abnormalities in GAPDH and cell death. Under nonreducing conditions, oxidants induced oligomerization and insoluble aggregation of GAPDH via the formation of intermolecular disulfide bonds. Because GAPDH has four cysteine residues, including the active site Cys149, we prepared the cysteine-substituted mutants C149S, C153S, C244A, C281S, and C149S/C281S to identify which is responsible for disulfide-bonded aggregation. Whereas the aggregation levels of C281S were reduced compared with the wild-type enzyme, neither C149S nor C149S/C281S aggregated, suggesting that the active site cysteine plays an essential role. Oxidants also caused conformational changes in GAPDH concomitant with an increase in β-sheet content; these abnormal conformations specifically led to amyloid-like fibril formation via disulfide bonds, including Cys149. Additionally, continuous exposure of GAPDH-overexpressing HeLa cells to oxidants produced disulfide bonds in GAPDH leading to both detergent-insoluble and thiophilin-S-positive aggregates, which were associated with oxidative stress-induced cell death. Thus, oxidative stresses induce amyloid-like aggregation of GAPDH via aberrant disulfide bonds of the active site cysteine, and the formation of such abnormal aggregates promotes cell death.

In both prokaryotic and eukaryotic cells, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) plays a central role in glycolysis, catalyzing the reversible conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in a reaction that is accompanied by the reduction of NAD+ to NADH. Mammalian GAPDH is a homotetramer composed of four identical subunits. Recent studies show that mammalian GAPDH has diverse activities unrelated to its glycolytic function (1, 2), including roles in membrane fusion, microtubule bundling, nuclear RNA transport (2), regulation of Ca2+ homeostasis (3), and transcription (4). In addition to the various functions of GAPDH described above, particular attention is paid to its role in apoptosis (5–7). Although the proapoptotic role(s) of GAPDH seems to depend upon its accumulation in the particulate fractions, including the nucleus (5, 7), the detailed mechanism is still unclear.

Recently, it has been suggested that a wide variety of neurodegenerative diseases are characterized by the accumulation of intracellular and extracellular protein aggregates (8, 9). An initializing event in protein aggregation is thought to be the formation of an abnormal oligomer. For instance, β-amyloid and α-synuclein undergo conformational changes in Alzheimer disease and Parkinson disease, respectively, thereby acquiring a predominantly β-sheet structure that facilitates oligomer formation and subsequent amyloid fibril formation (10, 11). Formation of abnormal oligomers and amyloid fibrils in brain is likely to be induced by a variety of causes, including oxidative stress (8, 9, 12). It is also reported that an increase of oxidative stress in brain is well correlated with the progression of neurodegenerative diseases (13–15). Both biochemical and immunohistochemical studies have revealed the presence of oxidized proteins in some neurodegenerative diseases (16, 17), suggesting a role for oxidants in neurodegeneration. Therefore, the

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2 The abbreviations used are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 2-ME, 2-mercaptoethanol; MeSO2, dimethyl sulfate; NO, nitric oxide; NOR3, (±)-E-4-ethoxy-2-(E)-hydroxyimino)-5-nitro-3-hexenamide; SNGO, S-nitrosoglutathione; SNAP, S-nitroso-N-acetyl-L-cysteine; SIN-1, 3-(4-morpholino)pyridinium salt, hydrochloride; NOC18, 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene; DTT, dithiothreitol; BMH, bis-maleimidohexane; PBS, phosphate-buffered saline; mAb, monoclonal antibodies; pAb, polyclonal antibodies; BSA, bovine serum albumin; EGFp, enhanced green fluorescent protein; Mops, 4-morpholinepropanesulfonic acid.
abnormal oligomer and the resultant fibrils formed by oxidative stress might be an important component in the pathogenesis of these diseases. Indeed, oxidative modification of α-synuclein by either hydrogen peroxide (H$_2$O$_2$) or nitric oxide (NO) facilitated the formation of α-synuclein oligomers and aggregates, leading to neuronal cell death (18–21).

Protein cysteine residues are highly susceptible to various types of oxidation (22). Most mammalian GAPDHs have either three or four cysteines, including one at the active site (Cys$^{149}$), which are readily oxidized to sulfenic acid or sulfonic acid (23–26). Cysteine residues are also reported to play critical roles in protein aggregation, possibly initiated by the formation of stable cross-linked oligomers (27–29). These facts lead us to the hypothesis that oxidative stress may convert the normal conformation of GAPDH to an abnormal one via modification of the cysteines, resulting in aggregation. Indeed, GAPDH is deposited as insoluble aggregates in some neurodegenerative diseases, e.g., in senile plaques and neurofibrillary tangles in Alzheimer disease (30–32) and inclusion bodies (so-called Lewy bodies) in Parkinson disease (33). More recently, disulfide-bonded aggregates of GAPDH were observed in detergent-insoluble extracts from Alzheimer disease subjects (34). Thus, in this study, the molecular mechanisms underlying the oligomerization and aggregation of GAPDH induced by oxidants are investigated using purified GAPDH and in vitro oxidative stress. In addition, we analyzed whether aggregates of GAPDH possessed amyloid-like properties. Furthermore, we prepared GAPDH-overexpressing HeLa cells to investigate whether oxidative stress also produces abnormal GAPDH aggregates in the cells and affects the cell death. Here we report that oxidative stresses induce oligomerization and aggregation of GAPDH through aberrant disulfide bonds of the active site cysteine, readily leading to the formation of insoluble and amyloid-like aggregates. The implications of these results on cell death are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following reagents were obtained from the indicated commercial sources: rabbit GAPDH, rabbit malate dehydrogenase, and rabbit aldolase (Roche Applied Science); S-nitrosoglutathione (SNGO), (±)-(E)-4-ethyl-2,[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR3), S-nitroso-N-acetyl-dL-penicillamine (SNAP), 3-(4-morpholinyl)-sydnonimine, hydrochloride (SMI-1), 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC18), HilyMax transfection reagent, and 4,6-di-amidino-2-phenylindole dihydrochloride (Dojindo Laboratories, Kumamoto, Japan); anti-GAPDH mouse monoclonal antibody (anti-GAPDH mAb) (MAB374, Chemicon International Inc., Temecula, CA); anti-EGFP mouse monoclonal antibody (anti-EGFP mAb, JLI-8) and pEGFP-C1 (Clontech); anti-histone H2B goat polyclonal antibody (anti-histone pAb) (catalog number 07-371, Upstate Biotechnology, Inc., Lake Placid, NY); pBAD-His vector, Dulbecco’s modified Eagle’s medium, and antibiotics/antimycotics (Invitrogen); Sepharose 4B; Omniscript™ reverse transcriptase, QIAfilter™ plasmid midi kit, and the complete sequences were confirmed using a 373A DNA sequencer (PerkinElmer Life Sci-
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Expression and Purification of Recombinant GAPDH—For the expression of recombinant GAPDH proteins, expression vectors for the GAPDH gene were transformed into the gap (−) E. coli strain W3CG (generously provided from Prof. Pluckthun and Dr. Lindner at Zurich University) (36). The transformants were cultured for 2 h at 37 °C in M63 minimal medium containing both 50 μg/ml ampicillin and 15 μg/ml tetracycline, and then 0.2% (w/v) arabinose was added to the medium. After 24 h, the cells expressing recombinant proteins were collected by centrifugation (3000 × g) at 5 min at 4 °C and resuspended in lysis buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 30 mM imidazole, 10% glycerol, and 2 mM 2-mercaptoethanol (2-ME). The suspensions were sonicated on ice and centrifuged at 15,000 × g for 30 min (4 °C). The supernatants were incubated with nickel-nitrilotriacetic acid-agarose resin (50% slurry) for 2 h at room temperature with rocking. The resins were washed with 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, 50 mM imidazole, 10% glycerol, and 2 mM 2-ME. The proteins bound to the resin were eluted with 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, 300 mM imidazole, 10% glycerol, and 2 mM 2-ME, and the eluates were immediately mixed with 1 mM NAD<sup>+</sup>, 1 mM DTT, and 1 mM EDTA, followed by incubation at 4 °C overnight. The reduced proteins were directly loaded onto a Hi-Load 16/60 Superdex prep-grade column equilibrated with buffer-G2'. The fractions containing GAPDH were pooled and concentrated using Amicon Ultra-15 (Millipore Japan, Tokyo, Japan). The protein concentrations were determined spectrophotometrically assuming a ε<sub>280</sub> of 1.0.

Treatment of GAPDH with Oxidative Stress or a Cross-linker—Native or recombinant GAPDH (0.6 mg/ml) was treated with various concentrations of NOR3, a NO donor, in buffer-G2' at 37 °C for varying periods of time. NOR3 is stable in dimethyl sulfoxide (Me<sub>2</sub>SO) and decays with first-order kinetics to release NO in an aqueous buffer at physiological pH. To investigate the effects of other NO donors (SNGO and SNAP) or oxidizing reagents (SIN-1 and H<sub>2</sub>O<sub>2</sub>) on GAPDH, these oxidants (10 μM concentration) were evaluated in the same manner as NOR3. After treatment, the reactions were terminated by passing through a MicroSpin<sup>TM</sup> G-25 column (800 × g, 2 min) equilibrated with buffer-G2'. The samples were subjected to the following experiments. Bis-maleimidohexane (BMH), an irreversible sulfhydryl to sulfhydryl cross-linking reagent, was freshly prepared as a 36 mM stock solution in Me<sub>2</sub>SO. BMH (1 mM) was added to 100 μl of solution containing GAPDH (1 mg/ml), 20 mM sodium phosphate buffer (pH 7.4), 150 mM NaCl, and then the mixture was incubated at 4 °C in the dark. After 40 min, the reaction was quenched with 20 mM DTT.

SDS-PAGE—To prevent unmodified thiols from oxidation during electrophoresis, the solutions of GAPDH were further incubated with 1 mM iodoacetamide for 10 min at room temperature in the dark. After the alkylation of thiols, the samples were mixed with an equal volume of SDS-sample buffer containing 250 mM Tris-HCl (pH 6.8), 2% SDS, 30% glycerol, 0.01% bromphenol blue in the presence (reduced) or the absence (nonreduced) of 100 mM DTT, and then heated at 100 °C for 5 min. These samples were separated by 5–20% SDS-PAGE (DRC, Tokyo, Japan), and the gels were stained using Gel-Code Blue staining reagents.

In Vitro Turbidity of Solutions of GAPDH and Other Enzymes Containing the Reactive Cysteines—Native or recombinant GAPDH (0.6 mg/ml) was treated with NOR3 as described above, but the period of incubation with NOR3 was varied (1–48 h). To measure the turbidity of solutions derived from aggregated GAPDH, the absorbance at 405 nm was recorded using a VERSA Max microplate reader (Molecular Devices). To examine the effect of Congo red on the turbidity, preincubation with Congo red at a concentration of 200 μM was performed. Alternatively, the reaction mixtures were centrifuged (22,000 × g, 30 min), and the supernatants (soluble GAPDH) were collected. The pellets (insoluble GAPDH) were washed twice, resuspended in an equal volume of buffer-G2', and sonicated on ice for 10 s. The samples were subjected to either reducing or nonreducing SDS-PAGE as described above. The turbidities of other enzymes (alcohol dehydrogenase, malate dehydrogenase, and aldolase) containing active site cysteines were also measured in the same manner.

Circular Dichroism—After GAPDH (0.6 mg/ml) was treated with a control or 100 μM NOR3 for 1 h at 37 °C, the reaction mixture was desalted with a NAP-5 column, and the eluate was centrifuged at 22,000 × g for 30 min, providing cleared supernatant. The CD spectrum of GAPDH was measured with a spectropolarimeter model J-820 (Jasco, Tokyo, Japan). The temperature of the solutions in the cuvette was controlled at 37 °C by circulating water. The path length of the optical quartz cuvette was 1.0 mm for far-UV CD measurements at 200–250 nm and 10 mm for near-UV CD measurements at 250–300 nm. GAPDH was dissolved in buffer-G2' at a concentration of 0.1 to 0.2 mg/ml. Spectra were obtained as the average of 10 succes-
sive scans with a bandwidth of 2.0 nm. The data were expressed as molar residue ellipticity (θ).

**Thioflavin-S Binding-dependent Fluorescence**—This assay was carried out as reported previously (37). Thioflavin-S was dissolved in 20 mM Mops (pH 6.5) at a concentration of 3.2 mg/ml, filtered, and stored at −30 °C. Stock solutions were diluted 50-fold with same buffer before use and kept in the dark. Fifty μl of GAPDH sample (see above section) was mixed with 450 μl of thioflavin-S solution, and the fluorescence intensity was measured at an excitation wavelength of 430 nm and an emission of 482 nm using a fluorescence spectrophotometer F-2000 (Hitachi, Tokyo, Japan).

**Congo Red Birefringence**—This assay was carried out as reported previously (38). Aliquots (100 μl) of GAPDH (0.6 mg/ml), as is or treated with 100 mM NOR3 for 24 h at 37 °C, were added to 900 μl of Congo red solution (25 μg/ml in PBS). This mixture was incubated for 30 min at room temperature and then centrifuged at 15,000 × g for 30 min. The pellet was resuspended in 100 μl of milliQ water. The drops of this suspension were allowed to dry on a slide glass for 10 min. The birefringence was determined with an Eclipse LV100POL microscope equipped with a polarizing stage (Nikon, Tokyo, Japan).

**Electron Microscopy**—Aliquots of GAPDH solution were placed onto 200-mesh copper grids covered by carbon-stabilized Formvar film. After 10 min of incubation in a humid chamber at room temperature, the samples were negatively stained with 2% uranyl acetate. The specimens were observed using a JEOL 1200 CX transmission electron microscope (Tokyo, Japan). Birefringence was determined with an Eclipse LV100POL microscope equipped with a polarizing stage (Nikon, Tokyo, Japan).

**Cell Culture, Transfection, and Treatment with NOC18**—HeLa cells (human carcinoma) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics/antimycotics at 37 °C in 5% CO2. For transfection of GAPDH cDNA, ~5 × 105 HeLa cells were seeded in a 6-cm dish. After 24 h, the cells were transiently transfected with 1 μg of pFLAG-CMV2-GAPDH plasmid using Effectene (Qiagen) or HilyMax (Dojindo) transfection reagent according to the manufacturer’s protocols. To investigate the effect of oxidative stress, the cDNA-transfected cells were treated with either a control (0.1 n NaOH) or NOC18 at the indicated concentrations and maintained for a further 48 h.

**Cell Viability**—HeLa cells were seeded at 2 × 104/well in a 96-well plate, transfected, and treated with NOC18 in the same manner as described above. The cell viability was assessed by an 96-well plate, transfected, and treated with NOC18 in the same manner as described above. The cell viability was assessed by an

**Subcellular Fractionation**—After treatment with either a control or NOC18, the transfected cells were washed twice with PBS and then incubated for 5 min in ice-cold PBS containing 40 mM iodoacetamide to protect unmodified thiols from oxidation during fractionation. All the subsequent steps were performed at 4 °C. Cells were scraped in 500 μl of buffer-A containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl2, 0.05% Nonidet P-40, 0.5% Triton X-100, 40 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture. After 10 min, the suspensions of cells were mixed vigorously for 15 s, and the aliquots were collected as a total cell lysate. The remaining lysates were centrifuged at 800 × g for 5 min, and the pellets were collected. The pellets were then resuspended in 200 μl of buffer-B containing 10 mM Hepes-KOH (pH 7.4), 25 mM NaCl, 3 mM MgCl2, 300 mM sucrose, 40 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture, and washed three times by centrifugation (3,000 × g, for 10 min) followed by suspension in buffer. Following addition of 100 μl of buffer-B, the pellets were sonicated for 30 s, and finally obtained as particulate fractions (7). All samples were stored at −80 °C until use. Protein concentrations of the samples were determined by the Bradford assay (Bio-Rad).

**Immunofluorescent Microscopy**—After treatment with either a control or NOC18, the transfected cells were washed twice with PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature. The cells were incubated with 5% bovine serum albumin (BSA) in PBS at 10 min at room temperature to block nonspecific binding and permeabilized with PBS containing 0.1% Triton X-100 for 5 min. The cells were then incubated with an anti-FLAG mAb (1:1000) in 1% BSA/PBS overnight at 4 °C. After washing with PBST, the specific signal of FLAG tag was visualized by staining the cells with a Cy3-conjugated goat anti-mouse IgG antibody (1:5000) using a confocal scanning microscope (C1si-T2000-E; Nikon, Tokyo, Japan). The fixed cells were also stained with 0.02% thioflavin-S as described previously (33). For nuclear staining, the cells were labeled with 4',6-diamidino-2-phenylindole dihydrochloride (1 μg/ml) for 10 min. For quantification of cells with aggregates, six microscopic fields were selected at random, and the percentage of cells with aggregates was measured among at least 500 transfected cells.

**Western Blotting**—Both total cell lysates and fractionated proteins were mixed with an equal volume of SDS-sample buffer containing 0.25 M Tris-HCl (pH 6.8), 2% SDS, 30% glycerol, 0.01% bromphenol blue in the presence (reduced) or the absence (nonreduced) of 100 mM DTT, and then heated at 100 °C for 5 min. These samples were separated by 5–20% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). The membranes were incubated for 1 h with 5% BSA in PBST (0.05% Tween 20 and 0.02% Na2SO4 in PBS) to block nonspecific binding. The membrane was then incubated for 2 h at room temperature with an anti-GAPDH mAb (1:300), an anti-FLAG mAb (1:490), or an anti-EGFP mAb (1:1000), followed by incubation for 1 h at room temperature with a peroxidase-conjugated affinity-purified secondary antibody (Zymed Labsoratories Inc.). The membranes were also reprobed with a histone H2B pAb (1:5000). Detection was performed using ECL plus and HyperFilm, according to the manufacturer’s protocol (Amersham Biosciences). The intensity of the bands was measured using Scion image software.

**RESULTS**

**Oxidative Stress Induces Oligomerization of GAPDH through Formation of Intermolecular Disulfide Bonds**—GAPDH runs as a band of ~36 kDa upon nonreducing SDS-PAGE (Fig. 1A, lane 1). Treatment of GAPDH with NOR3, a NO donor, at a concentration of 10 μM for 10 min at 37 °C resulted in formation of three extra bands corresponding roughly to a dimer (~66, 68, and 76 kDa) (Fig. 1A, lane 2). Upon SDS-PAGE under reducing...
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A. effects of NOR3 or BMH on the electrophoretic mobility of GAPDH under reducing and nonreducing conditions are shown. GAPDH (0.6 mg/ml) was treated with either 0.1% Me2SO as a control or 10 μM NOR3 in Me2SO at 37 °C for 10 min. The samples were mixed with an SDS sample buffer in the presence (+) or absence (−) of 100 mM DTT for SDS-PAGE. In the cross-linking study, GAPDH (11 μg) was treated with 1 mM BMH, an irreversible sulfhydryl to sulfhydryl cross-linking reagent, at 4 °C for 40 min and mixed with SDS sample buffer in the presence of DTT. Each sample (10 μg) was subjected to 5–20% SDS-PAGE followed by staining with Gel-Code Blue. B. effects of various NO donors and H2O2 on the electrophoretic mobility of GAPDH under nonreducing conditions are shown. GAPDH (0.6 mg/ml) was treated with NOR3 (lane 2), SNGO (lane 3), SNAP (lane 4), SIN-1 (lane 5), or H2O2 (lane 6) at 37 °C for 10 min. All drugs were used at a concentration of 10 μM. Each sample (10 μg) was subjected to 5–20% SDS-PAGE. Lane 1 is the untreated control. C. GAPDH (0.6 mg/ml) was treated with the indicated concentrations of NOR3 at 37 °C for 10 min. Each sample (10 μg) was subjected to 5–20% SDS-PAGE under nonreducing conditions. D. mass of the GAPDH multimers determined from their mobility in 5–20% SDS-PAGE (the lane treated with 100 μM NOR3 in C). Double log calibration graph of retardation coefficients (slope) versus molecular masses of several protein standards (open squares) for nonreducing SDS-PAGE using the same condition as for the GAPDH species (filled circle). E. GAPDH (0.6 mg/ml) was treated without or with 100 μM NOR3 at 37 °C for the indicated times. Subsequent procedures were carried out as in A.

conditions, the NOR3-induced dimer was not observed (Fig. 1A, lane 4). Treatment of GAPDH with 1 mM BMH, an irreversible sulfhydryl to sulfhydryl cross-linking reagent, resulted in formation of the dimer, despite the presence of DTT (Fig. 1A, lane 5). These results suggest that NOR3 causes dimerization of GAPDH through formation of intermolecular disulfide bonds.

It is well known that NO donors such as SNGO and SNAP, peroxynitrite donors such as SIN-1, and hydroxyl radical donors such as H2O2 inhibit GAPDH activity (23–26). We further examined whether these oxidants affected the electrophoretic mobility of GAPDH under nonreducing conditions. At a concentration of 10 μM, all of the oxidants induced dimerization (Fig. 1B). Treatment with either NO gas or sodium nitroprusside also induced the formation of moderate amounts of GAPDH dimer (data not shown).

These results suggest that the disulfide bonding of GAPDH subunits can be induced not only by NO but also by other reactive oxygen species, i.e. oxidative stress. Thus, the effect of NOR3 as oxidative stress on GAPDH was further evaluated in the following experiments.

The concentration-dependent effect of NOR3 on the formation of oligomerization of GAPDH was examined next (Fig. 1C). Under nonreducing conditions, treatment of GAPDH with NOR3 at concentrations ranging between 1 and 10 μM resulted in formation of the dimer (average mass = 70 kDa) as noted above. In addition, higher concentrations (30–1,000 μM) of NOR3 induced the formation of trimers (118 kDa), tetramers (145 kDa), pentamers (188 kDa), and further multimers in a concentration-dependent manner (Fig. 1C and D). Thus, treatment with NOR3 induced the oligomerization of GAPDH. A time course study demonstrated that the oligomerization induced by NOR3 at 100 μM was detectable at 5 min, and the oligomer was maintained until 60 min after treatment with NOR3 (Fig. 1E). Similar results were also obtained with the other oxidants described above (data not shown). Taken together, these results clearly indicate that oxidative stress (such as treatment with NOR3) formed distinct and stable oligomers of GAPDH through intermolecular disulfide bond formation in vitro.

Oxidative Stress-induced Oligomerization of GAPDH Leads to Its Insoluble Aggregation—Dimers and oligomers of disease-associated proteins such as α-synuclein have been reported to act as a seed of the assembly of longer polymers that form insoluble aggregates (19, 21). To test the hypothesis that the oxidative stress-induced oligomerization of GAPDH leads to its
effects of NOR3 on Far-UV and Near-UV CD Spectra of GAPDH. The effect of DTT on NOR3-induced aggregation of GAPDH is shown. GAPDH (0.6 mg/ml) was treated with 100 μM NOR3 at 37 °C for 24 h. The reaction mixtures were centrifuged, providing the supernatants (soluble GAPDH) and the pellets (insoluble GAPDH). The pellets were resuspended in an equal volume of buffer-G2 and sonicated on ice. The samples were subjected to either reducing or nonreducing 5–20% SDS-PAGE by applying an equal volume of solution from either the supernatants or the pellets.

Cys149, the Active Site Cysteine of GAPDH, Plays an Essential Role in the Aggregation Induced by Oxidative Stress—To identify the cysteine responsible for the oxidative stress-induced aggregation of GAPDH, we constructed cysteine-substituted mutants of GAPDH by site-directed mutagenesis. Rabbit GAPDH has four cysteines per monomer at amino acid positions 149 (the active site), 153, 244, and 281 (Fig. 3A) (35). We substituted cysteine at these positions with serine or alanine, creating the single mutants C149S, C153S, C244A, C281S, and double mutant C149S/C281S. The purity of recombinant GAPDHs was assessed by >98% by SDS-PAGE (Fig. 3B). We investigated NOR3-induced aggregation of each GAPDH mutant (Fig. 3C). In the wild type, NOR3 (24-h incubation) induced aggregation in a similar manner to native GAPDH as shown in Fig. 2B. In both C153S and C244A, aggregation could be detected at low concentrations of NOR3 (~1 μM) and was enhanced at higher concentrations. In contrast, the levels of aggregation in C281S were reduced to 45% of wild type at 100 μM NOR3. Furthermore, treatment of C149S with NOR3 at 100 μM led to low levels of aggregation (5% of wild type). Finally, C149S/C281S showed a complete absence of aggregation. Next, we analyzed insolvabilities of these solutions by SDS-PAGE (Fig. 3D). As expected, the amount of insoluble GAPDH in each pellet was correlated with the aggregation level. These results demonstrate that both Cys149 and Cys281 are involved in NOR3-induced aggregation of GAPDH, and Cys149 plays an essential role.

Effects of NOR3 on Far-UV and Near-UV CD Spectra of GAPDH—To elucidate the conformational changes of NOR3-treated native GAPDH, CD spectra in both the far-UV (200–250 nm) and near-UV (250–300 nm) regions were measured.

FIGURE 2. Aggregation of GAPDH induced by treatment with NOR3 in vitro. A and B, time course (A) or concentration dependence (B) of the increase in turbidity of solutions of GAPDH in the presence of NOR3 are shown. GAPDH (0.6 mg/ml) was treated without or with 100 μM NOR3 at 37 °C for the indicated times (A) or treated without or with the indicated concentrations of NOR3 at 37 °C for 24 h (B). C, the reaction mixtures treated without or with the indicated concentrations of NOR3 at 37 °C for 24 h were centrifuged, providing the supernatants (soluble GAPDH) and the pellets (insoluble GAPDH). The pellets were resuspended in an equal volume of buffer-G2 and sonicated on ice. The samples were subjected to either reducing or nonreducing 5–20% SDS-PAGE by applying an equal volume of solution from either the supernatants or the pellets. D, concentration-dependent effects of DTT on NOR3-induced aggregation of GAPDH are shown. GAPDH (0.6 mg/ml) was treated with 100 μM NOR3 at 37 °C for 24 h in the absence or the presence of DTT at the indicated concentrations. The measurement of turbidity (left) and nonreducing 5–20% SDS-PAGE (right) were performed as described in A–C.
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When GAPDH was treated with NOR3 for 1 h at 37 °C, the absolute CD intensity increased between the wavelengths of 205–230 nm as compared with untreated GAPDH, suggesting augmentation of the secondary structure of GAPDH (Fig. 4A). The secondary structure content of GAPDH was calculated from the spectra by the method of Chen et al. (39). Untreated GAPDH was composed of 15% α-helix, 18% β-sheet, and 67% coil (Fig. 4A, lower panel). Upon treatment with NOR3, the β-sheet contents of GAPDH increased from 18 to 26%, whereas the coil content decreased from 67 to 56%, with a slight increase in the α-helical content from 15 to 18%. On the other hand, the near-UV CD spectrum of GAPDH exhibited cotton effects, with minima at 262, 268, 280, and 290 nm and maxima at 260, 265, 272, 288, and 296 nm, indicating an anisotropic environment of the aromatic side chains (Fig. 4B). However, these cotton effects were decreased by treatment with NOR3, indicating changes in tertiary structure. In the case of wild-type GAPDH, similar cotton effects were observed and also drastically decreased by treatment with NOR3 (Fig. 4C, panel A). In the case of C281S, however, the NOR3-induced decrease of cotton effects was smaller than that of wild-type GAPDH (Fig. 4C, panel B). Moreover, both C149S and C149S/C281S displayed no NOR3-induced decrease of cotton effects (Fig. 4C, panels C and D). Together, these results indicate that both the increase in β-sheet content and the conformational change of GAPDH are triggered by NOR3 treatment, and they suggest that these effects are mainly caused by a disulfide bond of Cys149.

NOR3-induced Aggregates of GAPDH Display Some Characteristics of Amyloid-like Fibrils—We subsequently investigated if GAPDH aggregates possessed any amyloid-like characteristics. We first measured the fluorescence intensity of thioflavin-S binding, which is one of the hallmarks of amyloid-like fibrils. In native rabbit GAPDH, NOR3 increased the fluorescence intensity of C281S but to a lesser extent (42% of wild type at 24 h). On the other hand, NOR3 barely increased the fluorescence intensity of C149S (5% of wild type at 24 h). Finally, C149S/C281S showed no increase in the fluorescence intensity, similar to the results obtained from the turbidity study in Fig. 3. Next, we investigated the effect of another amyloid-binding dye, Congo red, on the aggregation of GAPDH. The azo-dye Congo red binds preferentially to β-sheet containing amyloid fibrils and also specifically inhibits the oligomerization of amyloidogenic proteins (40). Pretreatment with Congo red (200 μM) significantly reduced the aggregation of native GAPDH treated with NOR3 to 20% of that of control at 24 h (Fig. 5B).

To further address whether aggregated GAPDH had formed amyloid-like fibrils, two morphological analyses were performed, Congo red birefringence and transmission electron microscopy. Birefringence upon staining with Congo red is used as a method for identification of amyloid in vitro and in vivo (10). Aggregated GAPDH exhibited Congo red binding under nonpolarized light (Fig. 5C, panel a). A portion of the sample also demonstrated typical orange-green birefringence under polarized light (Fig. 5C, panel b). The macromolecular structures formed by aggregated GAPDH were also examined using electron microscopy. In the case of NOR3-treated GAPDH, long helical fibrils were observed (Fig. 5D, panels a and b). The fibrils were ~10 nm in width (Fig. 5D, panel b), and occasional broad ribbons were observed, which appeared to be composed of parallel aligned filaments (Fig. 5D, panel c). On the other hand, only amorphous structures and no distinguishable fiber-like structures were observed in the control GAPDH sample collected at 24 h after incubation without NOR3 (Fig. 5D, panel d). Taken together, these results suggest that the insoluble aggregates of GAPDH formed by oxidants possess some properties of amyloid-like fibrils.

Effect of NOR3 on Aggregate Formation of Other Enzymes Containing Reactive Cysteines—Alcohol dehydrogenase, malate dehydrogenase, and aldolase also contain some cysteines in or near their active site, similar to GAPDH (25). Therefore, the...
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To assess whether the aggregate-prone cysteines of GAPDH identified in vitro are also relevant under intracellular conditions, HeLa cells were transiently transfected with GAPDH cDNAs of wild type, C281S, C149S, and C149S/C281S. Fig. 7A shows the levels of GAPDH protein in the total cell lysates. N-terminal FLAG-tagged GAPDHs were overexpressed at comparable amounts to endogenous GAPDH (Fig. 7A, upper panel). The efficacy of transfection of each mutant was also similar to that of the wild type (Fig. 7A, lower panel). We next determined the effective dose of NOC18, a continuous NO donor, on the cell viability. Treatment of mock-transfected HeLa cells with NOC18 for 48 h reduced the viabilities in a dose-dependent manner (Fig. 7B); the ED$_{50}$ of NOC18 was calculated as 1 mM. Next, we used NOC18 at 1 mM to determine whether oxidative stress formed amyloid-like aggregates of GAPDH in the cells. To do this, immunostaining with the antibody against FLAG tag (red) and a thioflavin-S staining (green) were performed (Fig. 7C, panel a). One day after transfection, cells were further exposed to either a control or 1 mM NOC18 for 48 h. Under the control-treated conditions, the wild type FLAG-GAPDH was diffusely distributed throughout the entire cytoplasm and thioflavin-S-negative. Treatment of the cells with NOC18 resulted in the formation of abnormal aggregates of FLAG-GAPDH in both the nucleus and the cytoplasm. Additionally, the aggregates were strongly stained with thioflavin-S, suggesting that oxidative stress induced the amyloid-like aggregation of GAPDH in cell as well as in vitro. The number of transfected cells with aggregates is summarized in Fig. 7C, panel b. Treatment of wild type-overexpressing cells with NOC18 produced a significant increase (10.5% of transfected cells) compared with that of control cells (0.9%). In C281S-overexpressing cells, NOC18 also induced the formation of the aggregates but to a lesser extent (6.5%). In contrast, in both C149S- and C149S/C281S-overexpressing cells with NOC18, cells with aggregates could not be detected (~1%). To investigate the characteristics of the cellular aggregate, particulate fractions (including detergent-insoluble materials) were collected by centrifugation and analyzed by Western blotting using an anti-FLAG antibody (Fig. 7D, panel a). Under nonreducing conditions (Fig. 7D, left panel), the wild type was highly aggregated by the NOC18 treatment. C281S also showed NOC18-induced aggregation, but the level was reduced compared with the wild type. There was no aggregation in both C149S and C149S/C281S. Under reducing conditions (Fig. 7D, right panel), as expected, the aggregates almost disappeared, but small amounts of the dimer in both wild type and C281S were observed. These results were largely identical to that obtained from in vitro studies. Thus, both Cys$_{149}$ and Cys$_{281}$ appear to be related to the formation of disulfide-bonded amyloid-like aggregates of GAPDH induced by oxidative stress, and Cys$_{149}$ also plays an essential role in the cell. Despite NOC18 treatments, the relative amounts of Cys-substituted mutants in the particulate fractions decreased corresponding to the level of aggregation (Fig. 7D, panel b). These results also suggest that GAPDH aggregation leads to insolubility, and the protein accumulates into the particulate fraction. Involvement of GAPDH Aggregation in Oxidative Stress-induced Cell Death—Finally, we asked whether oxidative stress-induced GAPDH aggregation is associated with cell death. Overexpression of each FLAG-GAPDH did not affect the viability of control-treated cells (Fig. 8A). When treated with NOC18, cell death in cells overexpressing wild type was significantly enhanced compared with mock-transfected cells (Fig.
In C281S-expressing cells, NOC18 also induced significantly enhanced cell death, but to a lesser extent (Fig. 8B). The degree of cell death in both C149S- and C149S/C281S-overexpressing cells was almost comparable with that in mock-transfected cells (Fig. 8B). These results indicate that overexpression of GAPDH causes the enhancement of oxidative stress-induced cell death, the degree of which appears to be dependent on the potency of GAPDH aggregation.

**DISCUSSION**

In this study, we reconfirmed that oxidative stress induces the oligomerization and aggregation of GAPDH through formation of the intermolecular disulfide bonds in *in vitro* study (Figs. 1 and 2), as reported previously (34). Furthermore, we obtained several lines of new evidence. We first identified both Cys149 and Cys281 as the aggregate-prone cysteine residues of GAPDH (Fig. 3). The conformational changes in the GAPDH structure accompanied with the increase of β-sheet contents were found to be critical to the mechanism of oxidative stress-induced aggregation of GAPDH (Fig. 4). We also detected amyloid fibril-like features in the resultant aggregates (Fig. 5) but not among other enzymes containing reactive cysteines (Fig. 6). Finally, we showed that exposure of GAPDH-overexpressing HeLa cells to oxidative stress induced the formation of aggregates of GAPDH in both the nucleus and the cytoplasm via disulfide bond formation, including Cys149 and Cys281 (Fig. 7), and that the degree of aggregation is correlated with that of the oxidative stress-induced cell death (Fig. 8). These results are compatible with previous studies in which β-amyloid induced the disulfide bonding and aggregation of GAPDH in either HT22 cells or rat primary cortical neurons (34) and in which overexpression of GAPDH in COS-7 cells promoted apoptosis concomitant with formation of Lewy body-like inclusions (33).

As shown in Fig. 3, the site-directed mutagenesis experiments revealed that the disulfide bonds via Cys149 and Cys281 played important roles in GAPDH aggregation induced by *in vitro* oxidative stress. Although the aggregation level of C281S was moderate compared with the wild type, both C149S and C149S/C281S displayed almost no aggregation under oxidative stress (Fig. 3). Moreover, similar results were obtained from the cell culture studies (Fig. 7). Therefore, we conclude that the
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Cys^{149}–Cys^{149} disulfide, rather than Cys^{281}–Cys^{281}, plays an essential role in oxidative stress-induced GAPDH aggregation. Cys^{149} is the active site cysteine and is reported to be completely conserved in all mammalian GAPDH sequences, including human (41). Indeed, we have found that oxidative stress increased the turbidity of solutions, including human GAPDH, and that the substitution of Cys^{151}, the active site corresponding to Cys^{149} in rabbit GAPDH, to alanine or serine (C151A and C151S) diminished the oxidative stress-induced aggregation both in vitro and in cell (see supplemental figure). Thus, oxidative stress-induced aggregation of GAPDH via the active site cysteine may be a general event in most mammalian cells.

It is well known that the native form of rabbit GAPDH is composed of four identical monomers (termed O, P, Q, and R) and has no disulfide bonds in the interface and the folded structure (Fig. 3A) (37). The distances (Å) between the sulfur atom of Cys^{49} in each monomer were calculated using the analytical software CCP4mg to be: O–P, 29.9; O–R, 35.0; O–Q, 36.0; P–R, 36.1; P–Q, 35.0; and Q–R, 30.0. Therefore, in the native form each Cys^{49} cannot form a disulfide bond because of distance constraints. In addition, Cys^{149} is located at the bottom of the GAPDH active site (Fig. 3A and ref. 37). These facts lead to a possibility that oxidative stress may cause a conformational change in GAPDH, allowing access to another Cys^{149}, resulting in a paired Cys^{149} disulfide bond. This concept is compatible with our near-UV CD spectra of NOR3-treated GAPDH (Fig. 4). However, the mechanism for oligomerization and aggregation of GAPDH induced by oxidative stress seems to be more complex. NOR3 treatment resulted in higher order multicentric oligomers of GAPDH (in addition to 1–4 subunit species), indicating that not only Cys^{149} but also other cysteines could contribute to oligomer formation. In this regard, we propose a model of oxidative stress-induced oligomerization of GAPDH through formation of intermolecular disulfide bonds. First, oxidative stress causes a conformational change of GAPDH that allows a disulfide bond to form between Cys^{149} residues of different subunits. Subsequently, the
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The stable oligomer of GAPDH consisting of SDS-resistant molecules seems to be similar to that previously reported with α-synuclein (18, 19, 21) and β-amyloid (42, 43). It is well established that such disease-associated proteins exhibit some characteristics arising from their core structure, e.g. cross-β-structure, which involves both β-strands and β-turns (10, 42). In this study, far-UV CD spectra revealed that treatment with NOR3 increased the β-sheet content of GAPDH (Fig. 4) and that Congo red inhibited NOR3-induced aggregation (Fig. 5). Although we could not determine the precise structure of aggregated GAPDH, these results suggest the cross-β-structure transition. On the other hand, the near-UV CD spectra of NOR3-treated GAPDH clearly exhibited tertiary structural changes (Fig. 4). Therefore, the drastic changes in the anisotropic environment of the aromatic side chains, probably via Cys149-Cys149 disulfide bonds, might convert the folded structure into the β-sheet-rich aggregates, resulting in the insoluble fibrils. Indeed, several reports indicate that changes in disulfide bond formation can result in protein aggregation and amyloid-like fibrils (29, 44). In addition, some amyloid-like features of GAPDH aggregates were confirmed by morphological analyses (Fig. 5). To our knowledge, this is the first report to show such evidence. A variety of amyloids have filamentous structures with a width of ~10 nm and a length of 0.1–10 μm and high affinities to amyloid-binding dyes such as thioflavin-S and Congo red (10). The GAPDH fibrils also exhibit these typical features, and also had rare Congo red birefringence (Fig. 5). The slight difference between other amyloid fibrils and GAPDH fibrils may depend on the conditions of incubation, especially pH and ionic strength (45), or may be intrinsic characteristics of GAPDH. Comparative studies between typical amyloids such as a β-amyloid and that of GAPDH are in progress.

Continuous exposure to oxidative stress resulted in the appearance of DTT-insensitive oligomers of GAPDH both in in vitro and in cells (Figs. 2 and 7). These facts suggest another mechanism of aggregation, in addition to intermolecular disulfide bonds. As described above, Cys281 is an auxiliary residue for NOR3-induced aggregation of GAPDH. In contrast to the other three cysteines (Cys149, Cys153, and Cys244), Cys281 is outwardly oriented and located in a loop region (amino acids 275–288) (Fig. 3A). Earlier reports concerning the subunit interaction of GAPDH demonstrated that Cys281 in the loop region played a role in maintaining GAPDH structure (46, 47). Thus, we have hypothesized that the interaction between the loop regions in each subunit of GAPDH is also important in the formation of oligomers and aggregates. The formation of DTT-insensitive oligomers of GAPDH may be caused by such interactions.

Similar to the results obtained in vitro, we found that continuous exposure of GAPDH-overexpressing HeLa cells to NOC18, a continuous NO donor, produced disulfide-bonded aggregates of GAPDH in both the nucleus and the cytoplasm (Fig. 7 and supplemental figure). In addition, the aggregates were thioflavin S-positive (Fig. 7), suggesting that the aggregates are amyloid-like. Because such materials could be not detected in either C149S- or C149S/C281S-expressing cells, the disulfide bond via Cys149 is essential in the intracellular formation of amyloid-like fibrils. These findings are compatible with the results obtained from previous studies in which disulfide-bonded aggregates of GAPDH were found in detergent-insoluble extracts from Alzheimer disease subjects (34), and in which immunohistochemical examination of Parkinson disease subjects displayed nuclear aggregation of GAPDH in the affected neurons (33). We also found that NOC18 induced the endogenous GAPDH aggregation in mock-transfected cells, but the aggregates were thioflavin S-negative (data not shown). Therefore, not only disulfide bond assisted aggregation but also the overexpression of GAPDH in cells might be critical for GAPDH amyloidogenesis. Indeed, most previous reports have suggested that the overexpression of GAPDH and its subsequent translocation in particulate fractions appeared to be implicated in the initiation of cell death and also in the etiology of some neurodegenerative diseases (5–7, 30, 32–34). Interestingly, the participating amounts of Cys-substituted mutants in the particulate fractions were dependent on the level of its aggregation (Fig. 7). These results support the idea that the accumulation of GAPDH aggregates into the particulate fractions is determined by the conformational changes induced by oxidative stress.

In this study, we demonstrated that the toxic role(s) of GAPDH in oxidative stress-induced cell death depend on its
degree of aggregation. There are, however, some important questions regarding the aggregation and amyloid-like fibril formation of GAPDH induced by oxidative stress. First, what protein species are harmful to cells? It is well known that aggregated β-amyloid or α-synuclein and their amyloid fibrils are very toxic both extracellularly and intracellularly (8, 9). On the other hand, there is increasing evidence that several kinds of partially folded intermediates in the aggregation process, namely the oligomers and so-called protofibrils, are possibly toxic, rather than amyloid-like fibrils (8, 9). More recently, Nagai et al. (48) reported that the soluble β-sheet monomer of polyglutamine protein caused cytotoxicity, suggesting that the toxic conformational changes in the monomer are possibly responsible for neurodegeneration. In this regard, further investigations are required to determine the toxic protein species in the process of oxidative stress-induced aggregation of GAPDH.

A second question is as follows: how is the cell death mechanism of GAPDH aggregation associated with the NO-S-nitrosylation-GAPDH-Siah1 cascade, which is already proposed as a convincing mechanism of GAPDH-dependent cell death? Hara et al. (5) reported that S-nitrosylation of Cys156 of GAPDH in HEK293 cells (corresponding to Cys149 in rabbit) enhanced its binding with Siah1, leading to nuclear translocation and cell death. Therefore, this study could not exclude the possibility that the relief of NOC18-induced cell death in Cys149-substituted mutants resulted from the disappearance of GAPDH interactions with Siah1. However, we have observed a high correlation between GAPDH aggregation and cell death enhanced by oxidative stress (Fig. 8). Furthermore, we could not detect the S-nitrosylation of aggregated GAPDH treated by NOR3 in vitro (data not shown). Thus, not only the interactions of GAPDH with Siah-1 but also the GAPDH aggregations are associated with oxidative stress-induced cell death, and each cascade might operate independently.

In conclusion, our results indicate that oxidative stresses trigger a conformational change in GAPDH and induce aberrant disulfide bonds via the active site cysteine, resulting in the formation of amyloid-like fibrils and cell death. This study provides new insights into the roles of GAPDH in cell death, which has been implicated in the pathogenic mechanisms of neurodegenerative diseases.

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