Oxidation of an Exposed Methionine Instigates the Aggregation of Glyceraldehyde-3-phosphate Dehydrogenase

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Andre L. Samson1,2, Anja S. Knaupp3, Itamar Kass4, Oded Kleinfeld5, Emilia M. Marijanovic5, Victoria A. Hughes5, Chris J. Lupton6, Ashley M. Buckle5, Stephen P. Bottomley5, and Robert L. Medcalf3

From the 1Australian Centre for Blood Diseases, Monash University, Melbourne 3004, Victoria, Australia and 4Department of Biochemistry and Molecular Biology, 4Australian Regenerative Medicine Institute and Department of Anatomy and Developmental Biology, and 5Victorian Life Sciences Computation Centre, Monash University, Clayton 3800, Victoria, Australia

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a ubiquitous and abundant protein that participates in cellular energy production. GAPDH normally exists in a soluble form; however, following necrosis, GAPDH and numerous other intracellular proteins convert into an insoluble disulfide-cross-linked state via the process of “nucleocytoplasmic coagulation.” Here, free radical-induced aggregation of GAPDH was studied as an in vitro model of nucleocytoplasmic coagulation. Despite the fact that disulfide cross-linking is a prominent feature of GAPDH aggregation, our data show that it is not a primary rate-determining step. To identify the true instigating event of GAPDH misfolding, we mapped the post-translational modifications that arise during its aggregation. Solvent accessibility and energy calculations of the mapped modifications within the context of the high resolution native GAPDH structure suggested that oxidation of methionine 46 may instigate aggregation. We confirmed this by mutating methionine 46 to leucine, which rendered GAPDH highly resistant to free radical-induced aggregation. Molecular dynamics simulations suggest that oxidation of methionine 46 triggers a local increase in the conformational plasticity of GAPDH that likely promotes further oxidation and eventual aggregation. Hence, methionine 46 represents a “linchpin” whereby its oxidation is a primary event permissive for the subsequent misfolding, aggregation, and disulfide cross-linking of GAPDH. A critical role for linchpin residues in nucleocytoplasmic coagulation and other forms of free radical-induced protein misfolding should now be investigated. Furthermore, because disulfide-cross-linked aggregates of GAPDH arise in many disorders and because methionine 46 is irrelevant to native GAPDH function, mutation of methionine 46 in models of disease should allow the unequivocal assessment of whether GAPDH aggregation influences disease progression.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a ubiquitous intracellular oxidoreductase enzyme best known for its role in glycolysis (1). Beyond this metabolic function, GAPDH participates in many other diverse cellular processes, including microtubule bundling (2), apoptosis (3–5), and transcriptional (6) and post-transcriptional gene regulation (7).

Under basal conditions, GAPDH resides in the cytosol as an abundant (~1–15 g/liter) and highly soluble homotetramer (1, 8). In addition, GAPDH shuttles from the cytosol into other subcellular compartments in a regulated fashion. For example, GAPDH readily translocates into the nucleus during cytotoxic stress (3, 9–11).

In vivo studies show that GAPDH converts from its native soluble state into a non-native high molecular weight insoluble state during disease (11–20). For instance, insoluble aggregates of GAPDH have been observed in the affected tissues of patients with Alzheimer disease (13) and alcoholic liver cirrhosis (12). Interestingly, GAPDH is also a susceptibility locus for late onset Alzheimer disease (21). Furthermore, robust aggregation of GAPDH has been detected in rodent models of motor neuron disease (17) and methamphetamine abuse (11). However, whether GAPDH aggregation is a causative factor of disease remains to be determined.
Current hypotheses of GAPDH aggregation stipulate an essential role for the oxidation and disulfide cross-linking of its cysteine residues (11, 13, 22, 23). Indeed, GAPDH aggregates formed in vivo (11–13, 18) and in vitro (10, 11, 18, 22, 23) have been shown to be disulfide-cross-linked. Recently, we discovered another instance where GAPDH converts into an insoluble disulfide-cross-linked state (18). We found that neurotrauma in vivo or necrosis in culture caused GAPDH and many other intracellular proteins to convert into a disulfide-cross-linked high molecular weight insoluble state (18). The term “nucleocytoplasmic coagulation” was given to the injury-induced en bloc conversion of soluble intracellular proteins, including GAPDH, into a disulfide-cross-linked insoluble state (18).

Here, as a model of nucleocytoplasmic coagulation, we investigated the mechanism by which free radicals trigger GAPDH aggregation in vitro. We confirmed that GAPDH aggregation involves intermolecular disulfide cross-linking. However, our data suggest that disulfide cross-linking is a secondary step in GAPDH aggregation. Instead, we found that free radical-induced oxidation of a surface-exposed methionine (Met-46) is a primary event that allows GAPDH misfolding and subsequent disulfide cross-linking and aggregation to occur. Mutation of Met-46 rendered GAPDH resistant to free radical-induced aggregation, a strategy that should now be used in vivo to provide unequivocal insight into the influence of GAPDH aggregation on disease. In addition, our methodology and the elucidated mechanism of GAPDH aggregation provide a useful precedent for elucidating the basis of nucleocytoplasmic coagulation and other free radical-induced protein aggregation events.

EXPERIMENTAL PROCEDURES

Materials—Unless stipulated all reagents were from Sigma-Aldrich. NOR3 was from Cayman.

Site-directed Mutagenesis of GAPDH—N-terminally His6-tagged human wild-type GAPDH in the pET-14b vector was kindly provided by Prof. J. J. Tanner (University of Missouri-Columbia). Residues were mutated using the QuikChange site-directed mutagenesis approach (Stratagene), and all mutations were verified by DNA sequencing. All mutations were conservative in nature (according to the PyMOL Molecular Graphics System v1.5.0.4 mutagenesis predictor, Schrödinger, LLC) and involved the introduction of a residue with a lower reactivity toward oxidative modification than the corresponding wild-type residue. Lastly, AmylPRED2 analysis (24) was performed to ensure that overt aggregation-prone regions were not altered/created by mutation (data not shown).

Expression and Purification of Recombinant Human GAPDH and Its Mutants—The expression and purification of wild-type GAPDH and its mutants followed prior procedures (22) with minor modifications. In summary, protein expression was performed in BL21(DE3) C41 cells overnight at 28 °C in Overnight Express TB autoinduction medium (Merck). Cells were lysed in lysis buffer (300 mM NaCl, 30 mM imidazole, 10% glycerol, 1 mM β-mercaptoethanol, 100 mM sodium phosphate, pH 8.0), and the proteins were purified using a 1-ml HisTrap HP column (GE Healthcare). After elution from the nickel column, the samples were desalted into Buffer G2* (150 mM NaCl, 1 mM EDTA, 5% glycerol, 50 mM Tris-HCl, pH 8.0) and incubated overnight at 4 °C with 1 mM dithiothreitol and 1 mM NAD+.

Circular Dichroism and Thermal Denaturation—Circular dichroism (CD) measurements were performed on a Jasco J-815 CD spectrometer at 20 °C. Far-UV CD spectra (190–260 nm) were recorded in a 0.1-cm-path length quartz cuvette at a protein concentration of 0.5 mg/ml in 25 mM Tris-HCl, pH 8.0, 100 mM NaCl using a data pitch of 0.1 nm and a scan speed of 100 nm/min. The spectra for each protein were averaged across ≥3 independent experiments. To determine the effect of oxidation on near-UV and far-UV CD spectra, GAPDH (at 4 mg/ml) was incubated in Buffer G2 containing 667 μM NOR3 or DMSO as a control for hAt 37°C (a time point at which wild-type (WT) GAPDH aggregation just starts to occur). Samples were then buffer-exchanged into 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, and near-UV CD spectra (250–310 nm) were recorded in a 1-cm-path length quartz cuvette using a data pitch of 0.1 nm and a scan speed of 50 nm/min. The spectra were averaged across three independent experiments. After near-UV CD, the samples were diluted 1:8 with 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, and far-UV CD spectra were recorded as described. Thermal denaturation measurements (25–80 °C) were performed with the far-UV CD samples using a heating rate of 1 °C/min, and the change in signal at 222 nm was followed. To obtain the midpoint of denaturation (Tm), the thermal denaturation data were fit to a two-state unfolding model because the first derivative of the thermal denaturation curve yielded a single transition peak (data not shown).

GAPDH Enzymatic Activity Assay—100-μl reactions of 0.01 g/liter GAPDH, 813 μM D-glyceraldehyde 3-phosphate, and 198 μM NAD+ in buffer (1 mM EDTA, 0.1 M KCl, 10 mM K2HPO4, pH 8.9) were set up in a 96-well plate, and changes in absorbance (λ = 340 nm) were read continuously at 30 °C. Glycolytic activity was determined by performing linear regression analysis on the changes in absorbance over the first 2 min of incubation (with r2 > 0.98).

GAPDH Iodoacetamide Pretreatment—GAPDH preparations were diluted to 6 g/liter in ice-cold Buffer G2*. Samples were incubated with 2 mM tris(2-carboxyethyl)phosphine for 25 min at room temperature and then incubated in the absence/presence of 6 mM iodoacetamide for 25 min in the dark at room temperature. Samples were then buffer-exchanged into fresh ice-cold Buffer G2* using a 10-kDa-cutoff ultrafiltration...
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column (Millipore 0.5-ml Amicon Ultra; 4 × 3.5 min, 16,100 × g, 4 °C) and readjusted to a final concentration of 6 g/liter.

GAPDH Aggregation—For turbidity assays, 200-μl reactions of 0.6 g/liter GAPDH and 100 μM NOR3 (or an equivalent volume of DMSO as a vehicle control for NOR3) in Buffer G2’ were set up in an ice-cold 96-well plate (Nunc). Samples were then topped with 80 μl of mineral oil, and the absorbance (A = 405 nm) was measured every 5 min for 8 h at 37 °C on a ThermoMAX microplate reader (Molecular Devices). For immunoblotting, mass spectrometry, microscopy, and other end point assays, aggregation reactions of 0.6 g/liter GAPDH and 100 μM NOR3 (or an equivalent volume of DMSO as a vehicle control for NOR3) in Buffer G2’ were set up in ice-cold 1-ml microcentrifuge tubes and incubated at 37 °C for the stated period of time. For Thioflavin-T assays, 24-μl reactions of 1.5 g/liter GAPDH, 32 μM Thioflavin-T, and 250 μM NOR3 (or an equivalent volume of DMSO as a vehicle control for NOR3) in Buffer G2’ were set up in an ice-cold clear bottomed/black walled 384-well plate (Corning). Plates were sealed with foil, and fluorescence (λexcitation = 450 nm; λemission = 490 nm) was measured every 5 min for 24 h at 37 °C on a FLUOstar OPTIMA microplate reader (BMG Labtech).

SDS-PAGE, Immunoblotting, and Quantitation—SDS-PAGE, immunoblotting, and quantitation followed prior protocols (18). A 1:1000 dilution of mouse anti-His tag primary antibody (Sero tec) and a 1:5000 dilution of donkey anti-mouse IRDye 800CW secondary antibody (LI-COR Biosciences) were used.

Microscopy—Preparations of GAPDH were diluted in Buffer G2’ prior to microscopic examination. For differential interference contrast microscopy, samples were imaged on a Zeiss Axio Observer Z1 microscope equipped with a Zeiss Plan-NEOFLUAR objective (20× magnification, 0.4 numerical aperture, air immersion). The objective for bright field microscopy was a Zeiss Plan-Apochromat (63× magnification, 1.40 numerical aperture, oil immersion). For polarization microscopy, samples were first stained in 25 mg/liter Congo Red for 20 min before imaging on an Olympus BX61 microscope equipped with an Olympus UplanSApo objective (40× magnification, 0.75 numerical aperture, air immersion). For transmission electron microscopy, samples were adsorbed onto a carbon-coated grid, stained with 1% (w/v) uranyl acetate, and then imaged on a Hitachi H7500 microscope with an accelerated voltage of 40 kV. All micrographs were processed with ImageJ v1.47q software.

Mass Spectrometry—12.5 μg of GAPDH was diluted in 2 g/liter ammonium bicarbonate solution with 2.25 g/liter tris(2-carboxyethyl)phosphine to a final volume of 27 μl and incubated at room temperature for 5 min. 3 μl of 18 g/liter iodoacetamide was added, and samples were incubated for 20 min in the dark at room temperature. 1 μl of 200 mg/ml sequence grade trypsin (Promega) was added, and samples were incubated at 37 °C for 3 h followed by acidification with formic acid to quench trypsin activity. The resulting peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the QExactive mass spectrometer (Thermo Scientific, Bremen, Germany) coupled on line with a rapid separation LC nanoHPLC system (Ultimate 3000, Thermo Scientific, Bremen, Germany). Samples were loaded on a 100-μm, 2-cm nanoViper PepMap100 trap column in 2% acetonitrile, 0.1% formic acid at a flow rate of 15 μl/min. Peptides were eluted and separated at a flow rate of 300 μl/min on a Thermo rapid separation LC nanocolumn (75 μm × 15 cm, PepMap100 C18, 3-μm 100-Å pore size) using acetonitrile that was elevated from 2 to 8% over 1 min followed by a linear acetonitrile gradient from 8 to 24% in 0.1% formic acid for 14 min followed by a linear increase to 30% acetonitrile in 0.1% formic acid over 5 min and an additional increase up to 80% acetonitrile in 0.1% formic acid over 5 min followed by reduction of acetonitrile back to 2% and re-equilibration. The eluent was nebulized and ionized using the Thermo nanoelectrospray source with a distal coated fused silica emitter (New Objective, Woburn, MA) with a capillary voltage of 1.8–2.2 kV. The QExactive instrument was operated in the data-dependent mode to automatically switch between full-scan MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 375–1600) were acquired in the Orbitrap with 70,000 resolution (m/z 200) after accumulation of ions to a 3 × 106 target value with maximum injection time of 120 ms. Dynamic exclusion was set to 15 s. The 10 most intense multiply charged ions (z ≥ 2) were sequentially isolated and fragmented in the octopole collision cell by higher energy collisional dissociation with a fixed injection time of 60 ms, 17,500 resolution, and automatic gain control target of 1 × 105 counts. A 2.5-Da isolation width was chosen. The underfill ratio was at 10%, and dynamic exclusion was set to 15 s. Typical mass spectrometric conditions were as follows: spray voltage of 2 kV, no sheath and auxiliary gas flow, heated capillary temperature of 275 °C; normalized higher energy collisional dissociation energy of 27%, and subjected to LC-MS analysis.

Label-free Quantitation of Mass Spectrometry Data—Wild-type and cysteine-free GAPDH protein mass spectrometry data were analyzed using the Trans-Proteomic Pipeline version 4.6.3 (25). Trans-Proteomic Pipeline-processed centroid fragment peak lists in mzML format were searched against a database composed of UniProt Escherichia coli strain K12 (release date, January 8, 2013) supplemented with human WT and cysteine-free GAPDH sequences. The 4305 protein sequences were supplemented with their corresponding decoy sequences and sequences of known contaminants (the Common Repository of Adventitious Proteins (cRAP) database was downloaded in August 2013). The database searches were performed using X! Tandem with high resolution k-score plug-in through the Trans-Proteomic Pipeline. Search parameters included the following: trypsin or chymotrypsin cleavage specificity with two missed cleavages and cysteine carbamidomethylation as a fixed modification. Nitration/oxidation of tyrosine, methionine, and tryptophan and deamidation of asparagine and glutamine were set as variable modifications. Peptide tolerance and MS/MS tolerance were set at 20 ppm. X! Tandem refinement included semi-style cleavage. Peptide and protein lists were generated following Peptide Prophet and Protein Prophet analysis using a peptide false discovery rate of <1%. These modifications were selected based on a database search using Peaks Studio (version 6, Bioinformatics Solutions
fluctuations of backbone heavy atoms with respect to their initial structure were calculated every 20 ps (after performing a least square fit to their initial structure). Results were reported as the average root mean square fluctuation per residue across the simulation. For hydrogen bonding analysis, hydrogen bonds were calculated as a function of time. Two atoms were defined as having a hydrogen bond if the distance between the donor and acceptor atoms was ≤0.35 nm and the acceptor-donor-hydrogen angle was <30°.

In Silico Modeling of Intermolecular Disulfide Bond—Modeling of disulfide-linked GAPDH molecules was performed using PyMOL (The PyMOL Molecular Graphics System, v1.5.0.4). Two GAPDH molecules were spatially positioned to allow the in silico formation of a disulfide bond to position Cys-152. The complex was then inspected manually for clashes using PyMOL.

Statistical Analyses—Unless stipulated, all experiments were performed independently at least three times. Statistical analyses were performed using GraphPad Prism v6.01 software. The statistical analysis applied to each data set is stipulated in the accompanying legend.

RESULTS

Intermolecular Disulfide Bonding in GAPDH Requires a Prior Conformational Change—Previous studies indicate that cysteine oxidation and intermolecular disulfide bonding are essential for GAPDH aggregation (11, 22). Our modeling, however, suggests that the native conformation of GAPDH sterically prohibits disulfide bonds from forming (Fig. 1A). Hence, an initial conformational change in GAPDH most likely occurs before disulfide bonds can form.

Cysteine-free GAPDH and Wild-type GAPDH Have the Same Structure and Stability—To investigate whether disulfide-independent events also occur during GAPDH aggregation, we made a cysteine-free variant of GAPDH (via three cysteine-to-serine substitutions; C152S, C156S, and C247S). Characterization of the native structure of cysteine-free GAPDH by circular dichroism showed that native cysteine-free GAPDH and native wild-type GAPDH had the same overall secondary structure (Fig. 1B). Both native proteins also had equivalent thermal stability (Table 1). Lastly, because Cys-152 forms part of the glycolytic active site of GAPDH, we confirmed that cysteine-free GAPDH had a markedly reduced capacity to convert NAD⁺ to NADH in the presence of D-glyceraldehyde 3-phosphate (data not shown). Thus, although cysteine-free GAPDH had diminished glycolytic activity, it possessed a native structure and stability similar to those of wild-type GAPDH.

Non-Cysteine Residues Also Mediate GAPDH Aggregation—Previous studies show that free radical donors such as NOR3 cause cysteine-dependent aggregation of GAPDH (11, 22). We adopted the same methodology as these prior studies and treated GAPDH with NOR3 for 72 h. The resultant soluble and insoluble material was then subjected to SDS-PAGE analysis. As shown in Fig. 1C, NOR3 caused considerable amounts of wild-type GAPDH to convert into an insoluble state. Although a significant proportion of insoluble NOR3-treated wild-type GAPDH migrated at monomeric weight under non-reducing conditions, a considerable amount also migrated as high molec-
FIGURE 1. **Cysteine-independent events also mediate GAPDH aggregation.** A, molecular surfaces representation of native GAPDH homotetramer (with subunits colored yellow, cyan, green, and magenta). Cysteine 152 (colored red) is the most reactive cysteine that forms intermolecular disulfide bonds during GAPDH aggregation (11, 22). Shown are residues 136–169 (colored blue) from a different native GAPDH homotetramer modeled into a conformation suitable for disulfide bond formation; however, this positioning generates many steric clashes. B, far-UV circular dichroism spectra of WT-GAPDH (n = 6) and cysteine-free GAPDH (no cys-GAPDH; n = 5). C, 1660 μl of WT-GAPDH and cysteine-free GAPDH were treated with NOR3 for 72 h at 37 °C. Soluble (sup) and insoluble (pell) material were separated by centrifugation (16,100 g, 30 min). 0.5% of the soluble material and 3% of the insoluble material were boiled in SDS loading buffer 100 mM DTT and subjected to SDS-PAGE and Coomassie staining. An asterisk indicates the GAPDH monomer. Open arrowheads indicate DTT-resistant HMW SDS-stable aggregates. Arrows indicate very HMW SDS-stable aggregates. D, 650 μl of WT-GAPDH and cysteine-free GAPDH were incubated in the absence/presence of iodoacetamide (iaa) and then buffer-exchanged to remove unbound iodoacetamide. Proteins were then incubated with NOR3 (or DMSO as a vehicle control) for 20 h at 37 °C. Soluble material was isolated by centrifugation (16,100 × g, 30 min), and 10 μl were boiled in SDS loading buffer 0.5% dithiothreitol and subjected to quantitative anti-His tag immunoblot analysis. A representative immunoblot of the soluble monomeric GAPDH is depicted in pseudocolor to highlight quantitative differences in immunosignal (see Fig. 2A insert for the pseudocolor chart). The graph depicts the quantitation of the relative change in the amount of soluble monomeric GAPDH across n = 3 experiments (NOR3-treated samples were normalized to the corresponding DMSO-treated sample; white bars indicate the relative amount of soluble DMSO-treated GAPDH, and gray bars indicate the relative amount of soluble NOR3-treated GAPDH). Mean ± S.E. (error bars) is shown. *, p < 0.05; **, p < 0.01 by one-way analysis of variance with Newman-Keuls correction. n.s. indicates non-significance. E, 650 μl of WT-GAPDH and cysteine-free GAPDH were incubated with NOR3 (or DMSO as a vehicle control) at 37 °C. Samples were diluted 1:10 and subjected to differential interference contrast (DIC) microscopy (E, panel i), bright field microscopy (E, panel ii), or polarization light microscopy after Congo Red staining (E, panel iii). Aggregates were diluted 1:2 and subjected to transmission electron microscopy (E, panel iv). Scale bars, 10 μm.
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TABLE 1

| Thermal stability of wild-type GAPDH (WT), cysteine-free GAPDH (No Cys), and other GAPDH variants |
|-------------------------------------------------------------|
| WT, cysteine-free, Y320F, W196F, M105S, and M46L-GAPDH were taken from 25 to 80 °C, and thermal denaturation was measured by far-UV circular dichroism. The midpoint of transition (T_m) was determined using a two-state unfolding functional.
| Number of independent experiments (n) is stated. The degree of statistical significance (p value) between the T_m of WT-GAPDH and the other GAPDH variants was determined by one-way analysis of variance with Bonferroni’s correction. |
| N/A, not applicable. |

| WT | No Cys | Y320F | W196F | M105S | M46L |
|----|-------|------|------|-------|------|
| Average T_m (°C) | 60.7 | 59.9 | 56.8 | 54.4 | 60.6 | 63.5 |
| SD (°C) | 0.2 | 0.2 | 2.7 | 0.2 | 0.1 | 2.1 |
| p value | N/A >0.9999 | 0.0284* | 0.0008b | >0.9999 | 0.1805 |

* p < 0.05.

Results denote that cysteine-independent interactions are important for GAPDH aggregation.

GAPDH Aggregates Have a Repetitive Underlying Architecture—We next visually compared the aggregates formed by wild-type and cysteine-free GAPDH. Low magnification microscopy showed that the aggregates of wild-type and cysteine-free GAPDH were comparable in both gross size and overall shape (Fig. 1E, panel i) with large, often branched amorphous looking aggregates of up to ~200 μm in diameter. Close inspection revealed that the aggregates of both wild-type and cysteine-free GAPDH were actually composed of repeating spherical units (Fig. 1E, panel i). Spherical units were similar in size (0.5–2 μm in diameter) and exhibited increased optical density (Fig. 1E, panel ii) and Congo Red birefringence (Fig. 1E, panel iii) along its periphery. An electron-dense substructure running parallel to the circumference of each spherical unit was also discernible via transmission electron microscopy (Fig. 1E, panel iv). However, in contrast to prior reports (22, 38), no classic linear amyloid fibrils were observed during GAPDH aggregation. Thus, although the gross morphology of GAPDH aggregates appeared amorphous, each aggregate actually represented an assembly of repeating spherical units that harbored a distinct birefringent substructure. Most notably, the aggregates formed by wild-type and cysteine-free GAPDH were microscopically indistinguishable, supporting the notion that cysteine-independent mechanisms are important for GAPDH aggregation.

Cysteine Reactivity Does Not Principally Determine the Kinetics of GAPDH Aggregation—Next, the influence of cysteine reactivity on the rate of GAPDH aggregation was assessed. Immunoblot analyses of the insoluble fraction suggested that wild-type and cysteine-free GAPDH displayed a similar rate of NOR3-induced aggregation (Fig. 2A). To more accurately measure the rates of aggregation, wild-type GAPDH and cysteine-free GAPDH (with or without iodoacetamide pretreatment) were incubated with NOR3, and changes in solution turbidity were determined over time. As shown in Fig. 2B, the lag phase before increased turbidity was similar for both wild-type and cysteine-free GAPDH following NOR3 treatment (35 versus 30 min). The lag phase of aggregation was also unaffected by iodoacetamide pretreatment of wild-type and cysteine-free GAPDH (Fig. 2B). Similarly, the time to maximal turbidity was comparable for wild-type and cysteine-free GAPDH following NOR3 treatment (120 versus 130 min; Fig. 2B). Iodoacetamide pretreatment, however, did slightly prolong the time to reach maximum turbidity for wild-type GAPDH following NOR3 treatment (Fig. 2B). Altogether, these observations suggest that cysteine reactivity is not a major rate-determining step of GAPDH aggregation.

Iodoacetamide pretreatment, however, did significantly reduce the maximum turbidity achieved by wild-type GAPDH aggregation (Fig. 2B). Hence, cysteine reactivity does significantly affect the extent of GAPDH aggregation. Surprisingly, cysteine-free GAPDH aggregation resulted in an ~30% higher maximum turbidity value than that of wild-type GAPDH. This finding differs from our earlier observation that cysteine-free GAPDH aggregated to a lesser extent than wild-type GAPDH (Fig. 1D). The reason for this difference is unknown but may be due to the fact that...
changes in turbidity and changes in the amount of soluble GAPDH report on different aspects of aggregation.

For additional insight into the kinetics of GAPDH aggregation, we used Thioflavin-T, a dye that undergoes a defined Stokes shift when bound to amyloid-like aggregates (39). As shown in Fig. 2C, the changes in Thioflavin-T fluorescence during GAPDH aggregation largely mirror the previously noted changes in turbidity with 1) no overt difference in aggregation kinetics between wild-type and cysteine-free GAPDH, 2) iodoacetamide pretreatment reducing the magnitude but not altering the lag phase of wild-type GAPDH aggregation, and 3) cysteine-free GAPDH aggregation causing a greater fold change in Thioflavin-T fluorescence than wild-type GAPDH aggregation (Fig. 2C). Thus, although cysteine reactivity is a determinant of the extent of GAPDH aggregation, it plays only a subtle role in determining the rate of GAPDH aggregation.

**NOR3-induced GAPDH Aggregation Involves Met, Trp, and Tyr Oxidation**—To gain insight into the cysteine-independent means of GAPDH aggregation, we mapped the post-translational modifications that occur across GAPDH during NOR3 treatment. Accordingly, GAPDH was incubated with NOR3 for 20 h, then trypsin-digested, and subjected to LC-MS/MS (see supplemental File 1 for raw MS/MS files). MS/MS data were first analyzed using PeaksPTM for unbiased identification of post-translational modifications (26). PeaksPTM identified preferential oxidation of certain methionine and tryptophan residues in NOR3-treated GAPDH (data not shown). MS/MS data were then reanalyzed using the Trans-Proteomic Pipeline X! Tandem search engine (41). This search confirmed that NOR3 caused robust oxidation of specific Met, Trp, and Tyr residues in GAPDH (see supplemental File 2 for full X! Tandem peptide quantitation). For example, NOR3 caused reproducible oxidation of Met-46 of...
GAPDH (Fig. 3). In total, 15 residues were highlighted as pronounced sites of NOR3-induced oxidation (Fig. 4).

Label-free quantitation of post-translational modifications showed that, in the majority of instances, the same Met, Trp, and Tyr residues were oxidized to similar extents by NOR3 in both wild-type and cysteine-free GAPDH (Fig. 4). This finding confirms that wild-type GAPDH and cysteine-free GAPDH adopt comparable native conformations and suggests that they underwent similar structural rearrangements during NOR3-induced aggregation. Importantly, NOR3-induced modifications

![Mass spectrometry identifies methionine 46 as a prominent site of GAPDH oxidation.](image)
For each residue of interest, three independent parameters are tabulated: 1) the fold change in the percentage of oxidation after NOR3 treatment as derived from the experimental data presented in Fig. 4 and supplemental File 2, 2) the solvent-accessible surface based on the native GAPDH homotetramer (Protein Data Bank code 1u8f) and normalized to the solvent-accessible surface for the same residue of interest when situated within a Gly-X-Gly tripeptide, and 3) the contribution of the residue of interest to the thermodynamic stability of native GAPDH (Protein Data Bank code 1u8f) as predicted by glycine substitution and subsequent FoldX analysis (data are presented as ΔΔG in kcal/mol). The furthest right-hand column represents a combinatorial value predicting the likelihood of each residue of interest being the site of a primary oxidation and misfolding. Values were generated by multiplying the "fold change in percent oxidized" by the "solvent-accessible surface analysis data" and the "FoldX analysis data." Higher numbers predict a greater likelihood that the residue of interest is the site of a primary oxidation event. Note, as no peptides containing an oxidized Tyr-45 were detected in DMSO-treated GAPDH, a fold change in percent oxidized value was not possible for Tyr-45. Accordingly, the average fold change in percent oxidized value of 4.20 (i.e. obtained when averaging across all the other tabulated oxidation sites) was arbitrarily assigned to Tyr-45. SAS, solvent-accessible surface analysis; no Cys-GAPDH, cysteine-free GAPDH.

| Residue of interest | Fold change in percent oxidized peptides after NOR3 treatment averaged across WT- and no Cys-GAPDH | SAS normalized to Gly-X-Gly tripeptide | FoldX analysis: ΔΔG for glycine substitution for Protein Data Bank code 1u8f | Fold change × SAS × FoldX |
|---------------------|-------------------------------------------------|----------------------------------------|----------------------------------------|--------------------------|
| Tyr-42              | 2.91                                            | 0.029                                  | 17.39                                  | 1.472                    |
| Met-43              | 2.63                                            | 0.000                                  | 19.06                                  | 0.000                    |
| Tyr-45              | 4.20                                            | 0.083                                  | 18.97                                  | 6.577                    |
| Met-46⁠<sup>a</sup> | 6.51                                            | 0.071                                  | 16.79                                  | 7.707                    |
| Met-105<sup>a</sup> | 4.61                                            | 0.447                                  | 5.96                                   | 12.281                   |
| Met-130             | 2.02                                            | 0.117                                  | 11.61                                  | 2.739                    |
| Met-133             | 3.55                                            | 0.111                                  | 16.65                                  | 6.551                    |
| Met-175             | 2.80                                            | 0.005                                  | 20.48                                  | 0.313                    |
| Trp-196<sup>a</sup> | 6.48                                            | 0.311                                  | 10.19                                  | 20.519                   |
| Met-231             | 3.13                                            | 0.040                                  | 6.87                                   | 3.804                    |
| Trp-313             | 4.19                                            | 0.008                                  | 32.39                                  | 1.067                    |
| Trp-314             | 4.55                                            | 0.026                                  | 20.89                                  | 2.456                    |
| Met-320<sup>a</sup> | 12.28                                           | 0.004                                  | 15.54                                  | 8.370                    |
| Met-328             | 1.82                                            | 0.000                                  | 24.51                                  | 0.023                    |
| Met-331             | 1.68                                            | 0.005                                  | 23.08                                  | 0.189                    |

<sup>a</sup> The top four residues that were predicted to be primary oxidation events responsible for GAPDH aggregation.

occurred at discrete sites across GAPDH with pronounced oxidation at Tyr-45, Met-105, Met-175, Trp-196, Met-231, and Trp-313 but, for example, no increased oxidation of Trp-87 (Fig. 4). Although nitrosylation of Cys-152 was noted in aggregated GAPDH (data not shown), nitrosative adducts were scarcely detected elsewhere in GAPDH (e.g. 3–5% of Tyr-45- or Trp-313-containing peptides were nitrosylated after NOR3 treatment; supplemental File 2). Altogether, our results show that oxidation of specific Met, Trp, and Tyr residues occurs during NOR3-induced GAPDH aggregation.

A Method to Predict Initial Sites of Free Radical-induced Misfolding—Although at least one of the observed NOR3-induced modifications outlined in Fig. 4 may be a primary event that drives GAPDH aggregation, others are likely to be secondary events that contribute neither to the extent nor the rate of aggregation. To distinguish between primary and secondary events, we devised a ranking system where for each residue of interest the observed fold change in its oxidation during NOR3-induced aggregation was multiplied by both its solvent accessibility in native GAPDH and its importance to the thermodynamic stability of native GAPDH (see “Experimental Procedures” for details). This tripartite ranking system rests upon four key assumptions. 1) The primary oxidation event occurs when GAPDH is in its native homotetrameric state. 2) A large fold change in oxidation occurs for the primary event. 3) The residue of interest is important for the thermodynamic stability of GAPDH. 4) The primary oxidation event involves a solvent-accessible residue. This tripartite ranking system highlighted several NOR3-induced modifications in GAPDH as putative primary events (Table 2). In particular, oxidation of Trp-196, Met-105, Tyr-320, and Met-46 (mentioned in accordance with its ranking; see Table 2) were predicted as the four events most likely to instigate GAPDH misfolding and aggregation.

NOR3-induced Oxidation of Met-46 Drives GAPDH Aggregation—To experimentally test our predictions, the four highest ranked sites were conservatively substituted with residues that exhibit a higher resistance toward oxidative modification (42). The resultant GAPDH variants produced were M46L, M105L, W196F, and Y320F. Circular dichroism showed that all four GAPDH variants had the same overall secondary structure as native wild-type GAPDH (Fig. 5A). The GAPDH variants also possessed a similar thermal stability as wild-type GAPDH with the Y320F and W196F substitutions causing a slight but significant decrease in thermal stability and the M46L mutation causing a non-significant trend toward higher thermal stability (Table 1). Indicative of correct assembly into a native tetramer, all GAPDH variants exhibited an elution profile similar to that of wild-type GAPDH during size exclusion chromatography (data not shown). Thus, all four GAPDH variants possessed a similar native structure and a thermal stability comparable with that of native wild-type GAPDH.
Next, the influence of these mutations on the rate of NOR3-induced GAPDH aggregation was assessed. Changes in solution turbidity during NOR3 treatment showed that Y320F-GAPDH and wild-type GAPDH aggregated at the same rate/extent (Fig. 5B). A similar conclusion was reached when the NOR3-induced aggregation of Y320F-GAPDH was monitored using Thioflavin-T fluorescence (Fig. 5C).

A non-significant trend toward enhanced NOR3-induced aggregation for W196F-GAPDH was observed with higher turbidity (Fig. 5B) and Thioflavin-T fluorescence (Fig. 5C) achieved after NOR3-induced aggregation for W196F-GAPDH relative to wild-type GAPDH. The increased aggregation of W196F during NOR3 treatment likely relates to the fact that the W196F mutation seems to subtly destabilize the GAPDH molecule (Table 1).

By contrast, measurements of turbidity and Thioflavin-T fluorescence showed that M105L-GAPDH and M46L-GAPDH were significantly more resistant to NOR3-induced aggregation (Fig. 5, B and C). The M105L substitution reduced the extent but failed to alter the lag phase of NOR3-induced GAPDH aggregation. Hence, oxidation of Met-105 appears to be a secondary event that merely affects the extent of GAPDH aggregation.

The M46L substitution, however, dramatically reduced the extent and significantly prolonged the lag phase of NOR3-induced GAPDH aggregation. SDS-PAGE/immunoblot analysis of the soluble material after 72 h of NOR3 treatment confirmed that, of the four GAPDH variants, M46L was the only one highly resistant to aggregation (data not shown).

Based on these findings, we propose that oxidation of Tyr-320 and Trp-196 are bystander events that do not significantly affect NOR3-induced GAPDH aggregation. Oxidation of Met-105 is a secondary event that, akin to cysteine oxidation, causally contributes to the extent of GAPDH aggregation but does not principally determine its onset/rate. By comparison, oxidation of Met-46 represents a primary instigating event critical for the misfolding and aggregation of GAPDH.

**Methionine Oxidation Initiates GAPDH Aggregation**

**FIGURE 5. Comparing the native structure and aggregation of Y320F-, W196F-, M105L- and M46L-GAPDH.** A, far-UV circular dichroism spectra of WT-GAPDH and the top four predicted GAPDH variants (Y320F-, W196F-, M105L-, and M46L-GAPDH; n = 3 for each spectra). B and C, WT-, Y320F-, W196F-, M105L-, and M46L-GAPDH were incubated with NOR3 (NOR) (or DMSO as a vehicle control) at 37 °C. Aggregation over time was measured by changes in turbidity at $\lambda = 405$ nm ($n = 3$) and by relative changes in Thioflavin-T fluorescence ($n = 3$; all data were normalized to the maximum value reached by end stage aggregated WT-GAPDH). Data are mean $\pm$ S.E. (error bars). Two-way analysis of variance with Bonferroni’s correction for time versus protein treatment was performed. The maximum degree of statistical significance between NOR3-treated WT-GAPDH and each NOR3-treated GAPDH variant is indicated (n.s. indicates non-significance; *, $p < 0.05$; ***, $p < 0.001$). All NOR3-treated samples were significantly different ($p < 0.05$) from DMSO-treated samples (except for NOR3-treated M46L-GAPDH, which was not significantly different from DMSO-treated M46L-GAPDH).
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FIGURE 6. MD simulation of how methionine 46 oxidation affects GAPDH structure. A: representative root mean square fluctuation (RMSF) of one GAPDH subunit (the O subunit) indicating that oxidation of Met-46 (black line) increases the backbone fluctuations across the first ~120 N-terminal residues relative to non-oxidized GAPDH (red line). B and C, ribbon representations of the MD simulation performed on non-oxidized GAPDH (B) and oxidized Met-46 GAPDH (C). Only the region surrounding one Met-46 residue is shown with a superposition of 20 structures sampled every 7.5 ns from the last 150 ns of the simulations. Note that Met-46 (colored red) is located close to the interface between three subunits (namely the O subunit in blue, the Q subunit in gray, and the R subunit in gold) and its oxidation causes a local increase in the conformational plasticity across these three subunits. Supplemental Movie 1 depicts the MD simulations from which B and C are derived.

aggregation, we performed comparative MD simulations on human GAPDH with reduced or oxidized Met-46. Root mean square deviation analysis of backbone atoms during simulations showed that both systems reached equilibration (with plateau values of 0.16 ± 0.01 nm for GAPDH and 0.17 ± 0.01 nm for Met-46-oxidized GAPDH during 150 ns of productive simulation). Thus, Met-46 oxidation caused no gross change in GAPDH conformation or stability during simulation. Consistent with this notion, oxidized Met-46 did not form additional hydrogen bonds during simulation (0.04 ± 0.20 hydrogen bonds/ns) and did not significantly increase the overall solvent-accessible surface area of GAPDH (548 ± 5 nm² for GAPDH and 550 ± 5 nm² for Met-46-oxidized GAPDH).

Native GAPDH exists as an asymmetrical homotetramer comprising so-called O, P, Q, and R subunits (8). Although no gross alteration in GAPDH structure was noted upon Met-46 oxidation, root mean square fluctuation analysis of the individual subunits highlighted several discrete regions of Met-46-oxidized GAPDH that exhibited increased conformational variance. The most marked increase in conformational plasticity for Met-46-oxidized GAPDH occurred across residues 1–120 of the O subunit (Fig. 6A). Interestingly, Met-46 (colored red in Fig. 6, B and C) is situated at the interface of three subunits, and its oxidation not only increased backbone fluctuations within the O subunit (colored blue in Fig. 6, B and C) but also within adjacent regions of the Q and R subunits (colored gray and gold, respectively, in Fig. 6, B and C, and supplemental Movie 1). Based on these results, we postulate that Met-46 oxidation destabilizes nearby residues, increasing their solvent accessibility and their likelihood of oxidative modification. In turn, oxidation of these secondary sites likely leads to misfolding, disulfide cross-linking, and eventual aggregation of GAPDH. In support of this hypothesis, a dramatic increase in the solvent-accessible surface area of Tyr-45 (a residue robustly oxidized in aggregated GAPDH; Fig. 4) was observed in the MD simulations of Met-46-oxidized GAPDH (0.25 ± 0.07 nm² for GAPDH and 0.36 ± 0.14 nm² for Met-46-oxidized GAPDH; the higher standard deviation for Tyr-45 in Met-46-oxidized GAPDH is indicative of greater residue fluctuations).

DISCUSSION

There are numerous reports investigating the mechanism of GAPDH aggregation (11, 13, 22, 23, 43–47) with several studies stipulating an essential role for intermolecular disulfide bond formation (11, 13, 22, 23). These models of GAPDH aggregation are supported by the observation that insoluble disulfide-cross-linked forms of GAPDH have been found in vivo (11–13, 18). Our data suggest that cysteine oxidation contributes to GAPDH aggregation; however, we found that oxidation of non-cysteine residues has a more influential and primary role in GAPDH aggregation. In particular, our study suggests that oxidation of Met-46 is a crucial initial event for GAPDH misfolding and aggregation. MD simulations predict that Met-46 oxidation destabilizes nearby residues, increasing the likelihood of secondary oxidative damage. We predict that secondary oxidation events, including oxidation of Tyr-45 and Met-105, augment the misfolding of GAPDH, leading to intermolecular disulfide cross-linking and aggregation.

Our findings differ from prior work that shows that disulfide bond formation is a primary instigating event that drives free radical-induced GAPDH aggregation (11, 22). An instigating role for disulfide bond formation, however, seems highly implausible given that the narrowest distance between cysteine residues in the native GAPDH homotetramer (between Cys-152 and Cys-156 within the same subunit) is ~6.3 Å. Furthermore, these thiol groups point in opposite directions. This large Cys-to-Cys distance and the unfavorable side-chain orientation suggest that disulfide bond formation between the Cys-152 and Cys-156 would be highly unlikely without prior structural rearrangement of GAPDH. In silico modeling also suggested that the native conformation of GAPDH sterically prohibits Cys-152 from coming into close contact with any cysteine residue on another GAPDH subunit/homotetramer (Fig. 1A). Based on these factors, a preceding conformational change in GAPDH must occur before disulfide bonds can form. Thus, our model stipulating an initial misfolding event caused by oxidation of Met-46 provides a plausible explanation for how the cysteine residues of GAPDH might eventually be brought into close enough proximity to allow intermolecular disulfide bonding. Attempts to further dissect the initial oxidative misfolding of GAPDH using near-UV circular dichroism spectroscopy or thermal melt analysis were inconclusive.
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whereby NOR3-induced changes in the CD spectra and melting point of WT-GAPDH and M46L-GAPDH did not correlate with their vastly different aggregation propensities (data not shown).

The unique unitary composition of GAPDH aggregates suggests an atypical self-association mechanism that warrants further investigation. As wild-type GAPDH and cysteine-free GAPDH form aggregates that are microscopically indistinguishable (Fig. 1E), cysteine-based interactions are not responsible for the gross assembly of GAPDH aggregates. Notably, both cysteine- and non-cysteine-based aggregation events create binding sites for Thioflavin-T (Fig. 2C), and thus both are likely to be amyloid-like in nature. As with heat-denatured ovalbumin (48), the contribution of multiple concurrent aggregation events may explain why GAPDH aggregates do not form classic linear amyloid fibrils (Fig. 1E).

The mechanism of NOR3-induced GAPDH aggregation has implications for other protein aggregation events. For example, we recently found that GAPDH participates in nucleocytoplasmic coagulation, a novel aggregation event that involves the pronounced disulfide cross-linking of numerous intracellular proteins upon necrotic cell death in vivo (18). Studies are now addressing whether the oxidation of linchpin residues is a common event that precedes the disulfide cross-linking and aggregation of other intracellular proteins during nucleocytoplasmic coagulation.

It is well established that oxidation of redox-sensitive residues such as methionine, cysteine, and tyrosine can promote protein misfolding and aggregation (27). Here, we show that NOR3-induced GAPDH aggregation is a useful example of how oxidation of a surface-exposed “linchpin” residue can drive protein misfolding and aggregation. A similar sequence of events has been proposed for the aggregation of numerous other proteins that are structurally unrelated to GAPDH, including human growth hormone (49), transferrin (50), apolipoprotein A-I (52), interferon (53), huntingtin (54), e-casein (55), and γ-synuclein (56). Indeed, as free radical-induced protein misfolding is a widely accepted factor in aging and neurodegenerative disease (40), we anticipate that our novel tripartite ranking method can be used to expedite the identification of linchpin residues in other important free radical-induced protein aggregation events.

Insoluble aggregates of GAPDH are found in many diseases (11–19). No prior study has ascribed a structural-functional role for Met-46 in GAPDH biology. Met-46 of GAPDH is also widely conserved across the phylogenetic tree and does not form part of the glycolytic active site or NAD⁺ binding site. Hence, identification of Met-46 as a free radical-sensitive linchpin in GAPDH highlights an unequivocal means of addressing whether GAPDH aggregation influences disease progression. Future studies should now replace endogenous GAPDH with the aggregation-resistant glycoyltically competent M46L-GAPDH variant and determine whether this replacement alters disease etiology in mouse models of methamphetamine abuse, liver cirrhosis, and Alzheimer disease, instances where GAPDH aggregation has been mechanistically linked to cell death (11–13).

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